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Male reproductive dysfunction in *Solea senegalensis*: new insights into an unsolved question

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Abstract. Senegalese sole (*Solea senegalensis*) is a species with a high commercial value that exhibits a reproductive dysfunction in males born and raised in captivity (F_1) that hinders their sustainable culture. The present study evaluates the sperm quality and dopaminergic pathway of males born in the wild environment and of F_1 males. Traditional sperm analyses were performed, finding only significant differences in curvilinear velocity (VCL) and no significant differences in viability and total motility. No differences in global sperm methylation were observed either in spermatozoa or brain between the two groups (F_1 and wild-born males). However, our results point to a different sperm molecular signature between wild fish and fish born in captivity, specifically the differential expression in *miR-let7-d* and *miR-200a-5p* between these two groups. *miR-let7-d* has been correlated with spermatogenesis and sex preferences, whereas the miR-200 family is implied in target innervation of dopaminergic neurons in zebrafish. When we analysed the dopaminergic pathway, no differences were found in terms of different mRNA expression of dopaminergic markers. However, some differences were detected in terms of tyrosine hydroxylase protein expression by western blot analysis, thus suggesting an altered post-transcriptional regulation in F_1 males. The results of this study suggest that an altered sperm miRNA signature in F_1 males could be one possible mode of transmission of reproductive dysfunction to the progeny.

Additional keywords: dopamine signalling, miRNAs, molecular assays, mRNA, Senegalese sole, sperm quality.

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Introduction

The Senegalese sole is a species with a high commercial value whose culture has been hampered due to reproductive problems present in F_1 males (Guzmán *et al.* 2008). It has been pointed out that the absence of courtship is the main cause of reproductive failure, because breeders reared in captivity do not show the reproductive behaviour observed in wild breeders, namely prespawning chasing or paired synchronised spawning (Carazo *et al.* 2013). These studies demonstrated that wild males mated with females bred in captivity produced viable spawn, but non-viable spawn were obtained when wild females were mated with males bred in captivity (Mañanós *et al.* 2007). The absence of courtship may not be the only possible reproductive problem in this species.

It has been demonstrated that males born in captivity have lower fertilisation capacity than wild-born males (Forné *et al.* 2011). Some studies suggest that the low fertilisation rates obtained with captive Senegalese sole sperm samples could also be explained by low sperm volume (Chauvigné *et al.* 2017) and poor sperm quality (Cabrita *et al.* 2011). This could be the main reason for the low and variable fertilisation and hatching rates obtained after IVF using F_1 stripped eggs and spermatozoa (Rasines *et al.* 2012, 2013). It has also been reported that F_1 males have endocrine differences during the spawning period compared with wild-born animals (Guzmán *et al.* 2009; Chauvigné *et al.* 2016). These studies suggested the existence of a dopamine inhibitory tone in the brain–pituitary–gonadal axis regulating male reproduction in this species (Guzmán *et al.* 2009). Dopamine is a catecholamine implicated in many functions, including gonadotrophin inhibition via dopamine D2 receptors in fish (Vacher *et al.* 2000). Interestingly, in mice, dopamine released in the nucleus accumbens is involved in mediating sexual motivation (Beny-Shefer *et al.* 2017). In that study, the authors established a critical role for the mesolimbic dopaminergic system in governing pheromone-mediated responses and mate choice

in male mice (Beny-Shefer *et al.* 2017). Alterations in the dopaminergic pathway could be a key issue in the behavioural dysfunction of Senegalese sole F_1 males, as previously suggested by others (Guzmán *et al.* 2011; Carazo *et al.* 2013). Dopaminergic neurons of the central nervous system can be characterised by the coincident expression of a set of mRNAs collectively referred to as dopamine markers. These dopamine markers include tyrosine hydroxylase (*th*) as the rate-limiting enzyme in brain catecholamine biosynthesis, dopamine receptor 1 and 2 (*dr1* and *dr2*), transducin repeat-containing protein 2 (*trcp2*), vesicular monoamine transporter 2 (*vmat2*), dopamine transporter (*dat*), monoamine oxidase (*mao*) and catechol-*O*-methyl transferase (*comt*; Yamamoto and Vernier 2011).

The contribution of spermatozoa to the embryo is not limited only to DNA delivery during the fertilisation process; several transcripts and non-coding RNAs are also delivered to the embryo and could be crucial during and after early embryo development. The importance of the sperm RNA population in sperm quality and early embryo development has been explored in different species and recently accepted in mammals (humans) and fish (zebrafish) (Ostermeier *et al.* 2002; Meseguer *et al.* 2004; García-Herrero *et al.* 2011; Guerra *et al.* 2013; Valcarce *et al.* 2013). However, the specific functional activity of some of these sperm RNAs has yet to be elucidated. Although most studies at this level have been performed in mammals, particularly in humans, a set of RNA molecules has been associated with reproductive success in the fish, including brain-derived neurotrophic factor (*bdnf*), double-sex and mab-3 related transcription factor 1 (*dmrt1*) and FSHβ polypeptide (*fsh*), among others (Guerra *et al.* 2013).

Sperm non-coding RNAs, and in particular microRNAs (miRNAs), have also been described as crucial factors during early embryogenesis (Liu *et al.* 2012; Jodar *et al.* 2013) and changes in sperm miRNA can contribute to the transmission of certain phenotypes across generations (Dickson *et al.* 2018). The miRNAs are a conserved class of small non-coding RNAs that control gene expression by inducing mRNA degradation or by suppressing mRNA translation (Bartel 2004). Sperm miRNAs delivered in the embryo are considered important regulatory elements for early embryo transcriptional control (Wienholds *et al.* 2003). Some studies in mammals suggest that the presence of some miRNAs could be associated with sperm quality (Kotaja 2014). *miR-200a* and *miR-141* belong to the conserved miR-200 family, which has emerged as an important regulator of neurogenesis and sperm motility in zebrafish and humans (Xiong *et al.* 2018). In zebrafish, ectopic expression of *miR-200a* significantly reduced motility traits, namely curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP), and the fertilisation capacity of spermatozoa (Xiong *et al.* 2018). Moreover, *miR-let7-d* regulates the self-renewal of spermatogonial stem cells and spermatogenesis in the mouse (Niu *et al.* 2011).

Conversely, sperm DNA methylation status could be also relevant in sperm quality evaluation. In this context, alterations in DNA methylation have also been correlated with male infertility in humans (Gunes *et al.* 2016) and fish (Woods *et al.* 2018). DNA methylation is the most widely studied epigenetic mark that consists of the covalent binding of a methyl group to a DNA base (Labbé *et al.* 2017). The zebrafish is increasingly being used to study epigenetics in gametes (Wu *et al.* 2011; Jiang *et al.* 2013; Potok *et al.* 2013). The main characteristic of zebrafish spermatozoa is that the DNA is highly methylated (91–95% of cytosine–phosphorous–guanine (CpG) islands analysed; Jiang *et al.* 2013; Potok *et al.* 2013). High levels of DNA methylation in spermatozoa could also be expected in other teleost fish considering the high level of compaction of the sperm nucleus and the absence of transcription in the sperm cells.

In this study we maintained sole males born in captivity and males born in their natural environment for 18 months under the same conditions prior to the experiments. After this period, we analysed traditional sperm parameters, such as motility and viability, and confirmed that traditional sperm quality parameters did not differ significantly between the two groups. We analysed the presence of sperm mRNAs (*bdnf*, *dmrt1* and *fsh*), defined as molecular markers of reproductive success in fish, and three miRs (*miR-141-3p*, *miR-let7-d* and *miR-200a-5p*) present in teleost spermatozoa (Presslauer *et al.* 2017) and previously related to sex preferences or reproductive performance in human, zebrafish or mice (Beny-Shefer *et al.* 2017). We also analysed the global methylation status of spermatozoa from the different groups. Finally, an integrative analysis on the dopaminergic system was performed analysing brain dopamine mRNA markers and Th protein levels in wild- and captivity-born males.

Materials and methods

Ethics statement

All experiments were performed according to the directive of the Spanish and institutional bioethical guidelines of the Spanish Institute of Oceanography (IEO) and European Union Directive 2010/63/EU for the protection of animals used for experimental and other scientific purposes. The authorisation number for experimental procedures in this study is PI-10-16. Moreover, all people involved in the experiments have a Federation for Laboratory Animal Science Associations (FELASA) Class C permit for animal experimentation.

Animals

Wild and F_1 breeders of Senegalese sole were maintained for a period of 18 months in tanks with an open flow circuit, with a water renovation rate of 1.7 m^3 h^{-1} and constant moderate aeration. An artificial photoperiod of 16 h light and 8 h dark was used throughout the entire year. Temperature varied according to external conditions, maintaining thresholds of 21°C in the spring–summer months (May–September) and 12°C in the winter months (November–February). Wild males and a group of F_1 males born in captivity were fed with a natural diet for 3 days a week consisting of mussels, *Mytilus* spp., small squid, *Loligo* spp. and polychaete worms (*Nereis* spp.; Topsybaits). Both groups were fed a commercial diet (Vitalis Repro pellets; Skretting) for 2 days a week following the manufacture recommendations. The daily amount of food was adjusted taking into account the total biomass of the fish in the tank.

The biomass of the tanks was recorded monthly by individual samplings (size and weight).

Sample collection

Spermatozoa were collected from both wild-born males and F_1 males born in captivity. Samples were collected from December to the end of February, when breeders were maintained at mean ($\pm$ s.d.) temperatures of 12 ± 1°C. Individuals were anaesthetised with clove oil (40 p.p.m.) for 1.5 min, after which spermatozoa were collected by stripping, taking special care in order to avoid urine contamination. Sperm collection was performed as described previously (Cabrita *et al.* 2011). The urogenital pore was dried and spermatozoa were collected using a syringe by gently pressing the testes on the fish blind side. Samples were stored on ice in a Styrofoam support until further analysis; samples contaminated with urine were discarded.

To obtain brain samples, fish were immediately killed in the first 2 min by decapitation according to Chauvigné *et al.* (2017) and their brains were removed and stored in RNA Later (Thermo Fisher Scientific) at −80°C until determination of gene expression and western blot analysis.

Traditional sperm assays

Viability

Sperm membrane integrity was evaluated using propidium iodide (PI; Sigma) and 4′,6′-diamidino-2-phenylindole (DAPI) staining. PI was added at a final concentration of 1 μg mL^{-1} to assay cell viability based on membrane permeability. The total number of sperm cells was counted using 300 nM DAPI, a nuclear stain.

At least three microscope fields were photographed (Nikon Eclipse Ts2R), recorded and analysed using NIS-Elements image analysis software (Izasa Scientific) for each sample, for a total of 200 cells. The number of total and non-viable cells was counted and the percentage of viable cells calculated in each experimental group ($n = 6$ samples per group).

Motility

Motility analysis was performed by activating a 1-μL sample of spermatozoa with 5 μL seawater (temperature 21°C, salinity 35 p.p.t.).Total motility was determined using computer-assisted sperm analysis (CASA) and ISAS software (Proiser R+D). For all analyses, motility was assessed in a Makler chamber using a phase-contrast microscope (Nikon Eclipse Ts2R) with a ×10 negative contrast objective and a digital camera set at a rate of 50 frames per second (f.p.s.). The settings for the CASA software were adapted for this species. The following CASA parameters were measured: percentage of motile cells, VCL, VSL and VAP. Motility parameters were evaluated 30 s after sperm activation. Analyses were performed in sperm samples using 15 individual males from each of the wild and F_1 groups.

Sperm molecular assays

Global methylation

DNA was isolated from different sperm samples using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Sperm methylation status was evaluated in the two (wild and F_1) groups using the EpiJET DNA Methylation Analysis Kit (*Msp*I/*Hpa*II; Thermo Fisher Scientific). This kit uses the *Msp*I and *Hpa*II restriction enzymes to analyse DNA methylation. Epi *Msp*I and Epi *Hpa*II are isoschizomers with different sensitivities to CpG methylation. When the internal CpG in the 5′-CCGG-3′ tetranucleotide sequence is methylated, cleavage with Epi *Hpa*II is blocked, but cleavage with Epi *Msp*I is not affected. A simple digestion with *Msp*I and a simple digestion with *Hpa*II were performed for each experimental group for 1 h at 37°C. Moreover undigested DNA (as a negative control) and different controls with methylated and unmethylated DNA were included in the analysis. After the digestion, the reaction products were analysed by DNA electrophoresis on a 1% agarose gel. Three independent experiments of three different pools of males (each containing three males) were analysed.

Transcript and miR quantification

RNA and miR isolation. RNA and miRs were isolated from sperm samples of the two groups using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA quantity and purity were determined using a Nanodrop 1000 spectrometer (Thermo Fisher Scientific). Total RNA isolated was treated with DNase using the DNase I, RNase free kit (Thermo Fisher Scientific) for 30 min at 37°C to remove genomic (g) DNA contamination. The isolated RNA showed high purity (ratio of absorbance at 260 nm : 280 nm (A260/280) >1.8) and was stored at −80°C until further use.

Reverse transcription. For RNA sperm samples, cDNA was obtained from 1 μg RNA using a cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The reverse transcription (RT) conditions were 25°C for 5 min, 42°C for 30 min and 85°C for 10 min. For miR transcription, a specific TaqMan Small RNA probe (5×; Thermo Fisher Scientific) for each miR (*miR-200a-5p*, *miR-let-7d*, *miR -141-3p* and *miR-92a-3p*) was used according to the manufacturer's instructions. The RT conditions were 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. The cDNA of RNA and miR samples was stored at −80°C before quantitative (q) polymerase chain reaction (PCR) analysis.

Real-time PCR analysis. For transcript analysis, reactions were performed in a volume of 20 μL containing 10 μL SYBR green PCR master mix (Thermo Fisher), 2 μL primers (0.5 mM) and 2 μL cDNA template and made up to volume with molecular grade water. The amplification protocol used was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a final step at 95°C for 5 s. The primers and amplification protocols have been published previously for these transcripts (Guerra *et al.* 2013). Expression levels of each Senegalese sole transcript relative to the housekeeping gene elongation factor 1 alpha (*ef1α*; Infante *et al.* 2008) were calculated for all transcripts using the $2^{-\Delta\Delta Ct}$ method to analyse relative changes in gene expression. Because the $2^{-\Delta\Delta Ct}$ method requires the assignment of one housekeeping gene, which is assumed to be uniformly and constantly expressed in all samples, a housekeeping gene stability study was performed first. NormFinder software was used to evaluate candidate normalisation genes. NormFinder has the ability to measure the gene expression stability taking into account intra- and

intersample variations in defined groups. Results are expressed as the mean ± s.e.m. of the $2^{-\Delta\Delta Ct}$ calculated expression of three independent experiments of three different pools of males (each containing three males). Wild males were used as a reference group. For miR analyses, reactions were performed containing 10 μL TaqMan Universal PCR master mix II (Thermo Fisher), 2 μL primers (0.5 mM) and 2 μL product from the RT reaction, made up to volume with molecular grade water. The amplification protocol used was as follows: initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a final step at 95°C for 5 s. The specific probes and amplification protocols have been published elsewhere for these transcripts in mammalian spermatozoa (Abu-Halima *et al.* 2013; Kotaja 2014). Expression was normalised against that of *miR-92-3p* because of its high stability in previous miR expression studies in human spermatozoa (Corral-Vazquez *et al.* 2017) and because it is the most highly abundant miRNA in zebrafish spermatozoa (Presslauer *et al.* 2017). Three independent experiments of three different pools of males (each containing three males) were performed.

Target prediction

To analyse the potential targeted mRNAs of the miRs investigated (i.e. *miR-141-3p*, *miR-let7-d* and *miR-200a-5p*), we used TargetScanFish 6.2 (http://www.targetscan.org/fish_62/, accessed 5 October 2018; Peterson *et al.* 2014). TargetScanFish predicts targets by either the predicted efficacy of targeting (context + scores) or the probability of conserved targeting (P_{CT}). For conservation, the conservation of a 3′ untranslated region (UTR) is first determined, followed by analysis of a specific k-mer. Because one 3′UTR can contain multiple target sites, an aggregate P_{CT} is provided. For each type of k-mer, the number is provided for that target and whether it is considered a conserved site or a poorly conserved site (Peterson *et al.* 2014).

Brain molecular assays

Transcript quantification

RNA isolation. RNA was isolated from brain samples of the two groups using the TRIzol Isolation reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA quantity and purity were determined using a Nanodrop 1000 spectrometer (Thermo Fisher Scientific). Isolated total RNA was treated with DNase using the DNase I, RNase free kit (Thermo Fisher Scientific) for 30 min at 37°C to remove gDNA contamination. The isolated RNA showed high purity (A260/280 >1.8) and was stored at −80°C until further use.

Reverse transcription. cDNA was obtained from 1 μg RNA using the cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The RT conditions were 25°C for 5 min, 42°C for 30 min and 85°C for 10 min. cDNA samples were stored at −80°C before qPCR analysis.

Real-time PCR analysis. For transcript analysis, reactions were performed following the same protocol described for sperm samples. The primers and amplification protocols are given in Table 1. Expression levels of each transcript in Senegalese sole relative to the housekeeping genes *ef1α* and actin beta 2 (*actb2*) (Infante *et al.* 2008) were calculated for all transcripts using the $2^{-\Delta\Delta Ct}$ method to analyse relative changes in gene expression. NormFinder software was used to evaluate the candidate normalisation genes. Results are expressed as the mean ± s.e.m. of the $2^{-\Delta\Delta Ct}$ calculated expression of three independent experiments of six different males. Wild males were used as a reference group.

Th cloning

The complete open reading frame (ORF) from the *th* gene from *S. senegalensis* was cloned using standard procedures. Briefly, RNA was extracted from brain tissue using the Pure Link RNA Mini kit (Thermo Fisher Scientific) and reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific) using the protocols provided by the manufacturers. The oligonucleotides 5′-ACTCTGACACGCACAGATCG-3′ (forward) and 5′-AGGGGAAGCAAATAACAGCA-3′ (reverse) were used to amplify a 1548-bp fragment using the GoTaq DNA Polymerase (Promega) and subcloned in pCR4-TOPO vector (Thermo Fisher Scientific). To confirm the sequence of the cloned inserts, DNA sequencing was performed by the Servicio de Secuenciación (Sequencing Facility), Universidad de Salamanca, Spain. The sequences obtained were analysed using Chromas (Technelysium) and the translated amino acid sequence was automatically aligned against Th from *Homo sapiens* (*Homo*) NP_954987.2, *Mus musculus* (*Mus*) NP_033403.1, *Danio rerio* (*Danio*) NP_571224.1, *Esox lucius* (*Esox*) XP_010896475.1, *Oncorhynchus mykiss* (*Oncorhynchus*) XP_021419922.1, *Takifugu rubripes* (*Takifugu*) XP_003967406.1, *Oryzias latipes* (*Oryzias*) NP_001265797.1, *Nothobranchius furzeri* (*Nothobranchius*) XP_015820360.1 and *Xiphophorus maculatus* (*Xiphophorus*) XP_023199811.1 using Clustal Omega for Multiple Sequence Alignment (MSA) (https://www.ebi.ac.uk/Tools/msa/clustalo, accessed 5 October 2018; see Fig. S1, available as Supplementary Material to this paper).

Western blot analysis

S. senegalensis brains were mechanically homogenised with a PT 1200E Polytron homogeniser (Thermo Fisher Scientific) and insulin syringes and lysed in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS)) with protease inhibitors (Sigma-Aldrich). Samples were centrifuged at 10 000*g* for 10 min at 4°C to eliminate debris. The supernatants were transferred to clean Eppendorf tubes and protein content was determined by the Bradford method.

Lysates were resuspended in 2× Laemmli buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) and boiled for 7 min. Then, 100 μg total protein was separated using conventional SDS–polyacrylamide gel electrophoresis under reduced conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham). Membranes were blocked with 3% bovine serum albumin (BSA) in TBST (Tris buffer with 0.1% Tween 20; Sigma-Aldrich) for 1 h at room temperature and incubated with anti-Th antibody (1 : 1000 dilution in blocking buffer; AB152 (Lot NG1830749) Millipore) overnight at 4°C. After three washes with TBST for 10 min each time, membranes were incubated for

Table 1. List of primers used in molecular assays

Ta, annealing temperature; *dre-miR-141-3p*, *Danio rerio miR-141-3p*; *dre-miR-200a-5p*, *Danio rerio miR-200a-5p*; *dre-miR-92a-3p*, *Danio rerio miR-92a-3p*; hsa-miR-let-7d, *Homo sapiens miR-let-7d*; *Ss_actb2*, *Solea senegalensis actin b2*; *Ss_bdnf*, *Solea senegalensis brain-derived neurotrophic factor*; *Ss_comt*, *Solea senegalensis catechol-O-methyl transferase*; *Ss_dr1*, *Solea senegalensis dopamine receptor 1*; *Ss_dr2*, *Solea senegalensis dopamine receptor 2*; *Ss_ef1α*, *Solea senegalensis elongation factor 1*; *Ss_mao*, *Solea senegalensis monoamine oxidase*; *Ss_trcp2*, *Solea senegalensis transducin repeat-containing protein 2*; *Ss_vmat2*, *Solea senegalensis vesicular monoamine transporter 2*; *Zf_dat*, *Zebrafish dopamine transporter*; *Zf_dmrt1*, *Zebrafish double-sex and mab-3 related transcription factor 1*; *Zf_fsh*, *Zebrafish FSHβ polypeptide*; *Zf_th*, *Zebrafish tyrosine hydroxylase*

mRNA or microRNA	Sample	Reference	Oligonucleotide sequence (5′–3′)	Ta (°C)
Zf_dmrt1	Spermatozoa	Guerra *et al.* (2013)	Forward: ACGGGTCGCTGTCCATCA Reverse: GTGACACGAAGCCGTGGTTT	60
Ss_bdnf	Spermatozoa and brain	Self-designed	Forward: GACCAAGCCTATCTGGACCCGCAAC Reverse: TTGACAGCCAGGTGGTCCTCCCAGT	60
Zf_fsh	Spermatozoa	Guerra *et al.* (2013)	Forward: TGTGGAGAGCGAAGAATGTG Reverse: AGACCTTCTGGGTGTGCTGT	60
Ss_actb2	Spermatozoa	Infante *et al.* (2008)	Forward: AATCGTGACCTCTGCTTCCCCCTGT Reverse: TCTGGCACCCCATGTTACCCCATC	60
Ss_ef1α	Spermatozoa	Infante *et al.* (2008)	Forward: GATTGACCGTCGTTCTGGCAAGAAGC Reverse: GGCAAAGCGACCAAGGGGAGCAT	60
dre-miR-141-3p	Spermatozoa	miR database (MIMAT0001837)	TAACACTGTCTGGTAACGATGC	60
hsa-miR-let-7d	Spermatozoa	miR database (MI0000065)	TGAGGTAGTTGGTTGTATGGTT	60
dre-miR-200a-5p	Spermatozoa	miR database (MIMAT0031984)	CATCTTACCGGACAGTGCTGGA	60
dre-miR-92a-3p	Spermatozoa	miR database (MIMAT0001808)	TATTGCACTTGTCCCGGCCTGT	60
Ss_dr1	Brain	Self-designed	Forward: CGGCCAGCTATGTGATTCCA Reverse: TGTTTACCGTGCCCACTTCA	60
Ss_dr2	Brain	Self-designed	Forward: TCCAGGAGAAGGGTCACAGT Reverse: AAGGCCGAACAACAGAGGAC	60
Ss_trcp2	Brain	Self-designed	Forward: TGCTTTGCACCTTTATCGCT Reverse: GGGCTTTGGTCCTAATGGGG	60
Ss_vmat2	Brain	Self-designed	Forward: CCCCAGTCTAACAAACGGCT Reverse: AAGCATTCCCATTCCAGCCA	60
Zf_dat	Brain	Self-designed	Forward: TTCGCCACCTTCAATCCTCC Reverse: CCACCAGGTGATGGTCAGTC	60
Ss_mao	Brain	Self-designed	Forward: TGCAGGAGCTCTTCAACAGG Reverse: TCCACACTGCTTCACGTACC	60
Ss_comt	Brain	Self-designed	Forward: TTCTCAGTCCGACCCCTGAT Reverse: TGCTGAGCAGGATGCGTTTA	60
Zf_th	Brain	Self-designed	Forward: AGCAGCTCCACATCTTCCAC Reverse: AAACACGATCTGCTCGCTCA	62

1 h at room temperature with a secondary antibody, namely horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1 : 10 000 dilution; 111-035-003; Jackson ImmunoResearch). Membranes were washed again with TBST and the HRP signal was developed using an enhanced chemiluminescence (ECL) detection system (16101705; Advansta; WesternBright) in MicroChemi 4.2 (DNR Bio-Imaging Systems). The loading control was anti-β-actin antibody (1 : 1000 dilution in blocking buffer; 10/2016; Cell Signaling). Images were quantified using Adobe Photoshop CS6: total integrated density was obtained for each band, either Th or β-actin, and background integrated density was subtracted. To determine Th expression for each experimental group, the Th/β-actin ratio was calculated for each sample and was normalised against the mean of all WT samples using the following formula:

$$\text{Relative intensity}\,(Th/\beta-actin)=\frac{(Th/\beta-actin)_{meanF_1}}{(Th/\beta-actin)_{meanWild}}\times 100$$

Four different males were used per group (wild and F_1 males), and the results were analysed using two-tailed unpaired Student's *t*-tests in Graph Pad Prism version 5.

Statistical analysis

Data were analysed using SPSS v.22 (IBM Corp.) and Microsoft Excel. To determine the significance of differences between the two groups of male Senegalese soles in terms of sperm motility and viability, an independent samples *t*-test was use, with $P<0.05$ considered significant. Results are expressed as the mean percentage of all 15 individual males, whereas qPCR results for miR and transcript analyses in brains and spermatozoa are given as the mean ± s.e.m. of $2^{-\Delta\Delta Ct}$ calculated values. The Student's *t*-test ($\mu=1$) was used according to previous studies (Yuan *et al.* 2006) to identify changes in transcript levels between the control group (wild) and the study group (F_1). Three sperm pools (three males per pool) were analysed. Expression of Th was assessed in each and is expressed as

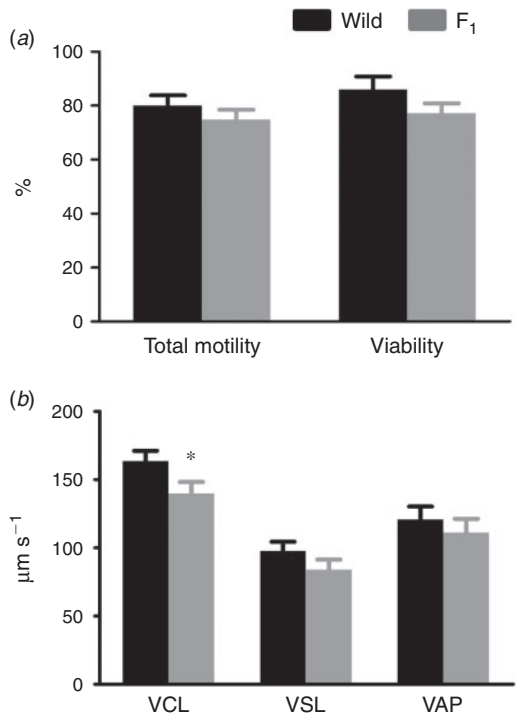

Fig. 1. Traditional analysis of sperm quality. (*a*) Total motility of spermatozoa, as analysed by computer-assisted sperm analysis (CASA), and viability of spermatozoa from male Senegalese sole (*Solea senegalensis*) born in a natural environment (wild) and born in captivity (F_1). There were no significant differences between the two groups. (*b*) Curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) in Wild and F_1 groups 30 s after sperm activation. Data are the mean ± s.e.m. *$P < 0.05$ compared with wild males. Analyses were performed in sperm samples from 15 individual males from each group.

a percentage of signal intensity over control (wild) values. Results were analysed using a one-sample *t*-test with Welch's correction. $P < 0.05$ was considered significant.

Results

Traditional sperm assays

Sperm from different groups showed high and similar initial motility (30 s after activation; Fig. 1*a*). When considering other parameters of sperm motility (VCL, VSL and VAP), sperm samples from F_1 males had a significantly lower VCL than samples from wild males 30 s after activation ($P < 0.05$; Fig. 1*b*). There were no significant differences in sperm viability between the two groups (Fig. 1*a*). Interestingly, there were no significant differences in traditional sperm parameters between the two groups except for the VCL parameter (Fig. 1).

Sperm molecular assays

Similar to what was observed in the analysis of traditional sperm parameters, global methylation analysis using the EpiJET DNA Methylation Analysis Kit (*Ms*pI/*Hpa*II) showed no differences between the wild and F_1 groups (Fig. 2*a*). A complete methylation status was observed in all sperm samples analysed, according to what could be expected in a normal transcriptionally inactive sperm cell with highly condensed DNA.

With regard to the mRNA molecular markers, there were no significant differences in the presence of the three studied mRNAs (*bdnf*, *dmrt1* and *fsh*) between the wild and F_1 groups (Fig. 2*b*). However, when the expression of miRs was analysed in sperm samples from the wild and F_1 groups, some differences were found. The expression of *miR-200a-5p* and *miR-let-7d* was significantly ($P < 0.05$) higher in F_1 compared with wild males, with a twofold increase in expression of *miR-200a-5p* (Fig. 2*c*).

mRNA target analysis using the TargetScanFish 6.2 program revealed that there are several potential mRNA targets of the miR-200 family that are involved in the dopaminergic pathway, namely *dr1*, *th*, *mao*, *comt* and *vmat2*. Specifically, analysis of *miR-200a* defined *mao*, *vmat2* and *comt* mRNAs as potential targets (Fig. S2).

Brain molecular assays

As in sperm cells, no significant differences were found between the F_1 and wild males in the global methylation assay of brain tissue (F1 *v.* wild males), with similar methylation patterns in brain samples from the two groups (Fig. 3*a*).

No significant differences were found in brain samples from F_1 and wild males in terms of *bdnf* and dopaminergic transcripts (dopaminergic transporters *dr1*, *dr2*, *trcp2*, *vmat2* and *dat*; degradation enzymes *mao* and *comt*; and dopamine biosynthetic enzyme *th*; Fig. 3*b*). Interestingly, when Th protein expression in the brain was analysed by western blot analysis, significant differences were found between the F_1 and wild groups: Th protein expression was reduced in F_1 males (mean ± s.e.m. 74.11 ± 5.77% vs 100.00 ± 2.96% normalised expression in wild males; Fig. 3*c*).

Discussion

S. senegalensis is a challenging species for aquaculturists because of a reproductive failure in F_1 males born and raised in captivity (Carazo *et al.* 2011). The inability of these animals to naturally procreate hinders the industrial large-scale take-off of the aquaculture of this species (Howell *et al.* 2009) doing necessary to use optimised artificial reproduction techniques for sustainable production (Riesco *et al.* 2017). Reproductive biologists have focused on this male reproductive failure due to the commercial potential of this species. Many publications have compared F_1 males with their wild counterparts at different levels, including comparisons of the transcriptome profile (Fatsini *et al.* 2016), sperm oxidative stress analysis (Valcarce and Robles 2016), behaviour (Carazo *et al.* 2013), hormone studies (Chauvigné *et al.* 2016) and sperm plasma composition (Forné *et al.* 2011). To gain an insight into the molecular status that may play a role in this reproductive problem, we used an integrative experimental design correlating cellular and molecular parameters within the brain–pituitary–gonadal axis. For this reason, we used two different samples: (1) spermatozoa, because they are the immediate products of the testicular environment and could be the carriers of altered patterns to the progeny; and (2) brain tissue. In this study, males from the two groups, namely those born in natural environments and those

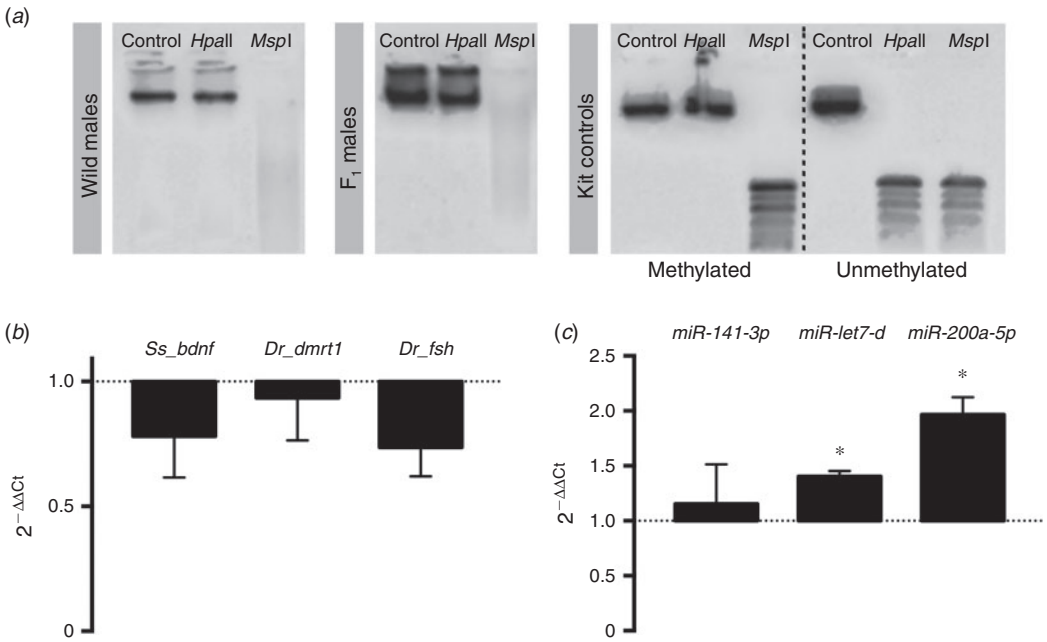

Fig. 2. Molecular analysis of sperm quality. (*a*) Global methylation status of sperm DNA, determined using an EpiJET DNA Methylation Analysis Kit (Thermo Fisher Scientific) from male Senegalese sole (*Solea senegalensis*) born in a natural environment (wild) and born in captivity (F_1). Three independent experiments of three different pools of three males per group were analysed (data not shown). Unmethylated plasmid DNA (kit control) is digested by both enzymes, whereas methylated plasmid DNA (kit control) is susceptible only to Epi *Msp*I digestion. (*b*) Expression levels for each gene relative to the housekeeping gene elongation factor 1 (*ef1α*) were calculated for all samples using the $2^{-\Delta\Delta Ct}$ method. The figure shows expression of each gene in the F_1 group relative to that in wild males, which was set to 1. (*c*) Expression of each microRNA normalised against that of *miR-92-3p* was calculated for all samples using the $2^{-\Delta\Delta Ct}$ method. The figure shows expression of each microRNA in the F_1 group relative to that in wild males, which was set to 1. Data are expressed as the mean ± s.e.m. of $2^{-\Delta\Delta Ct}$ values from three independent experiments with three replicates for each. $^*P < 0.05$ compared with the wild males.

born in captivity (F_1), that were analysed and compared were kept under an identical feeding regime and conditions (live diet plus fish feeds for breeders for a period of 18 months).

We first evaluated sperm motility in both groups. Nowadays, sperm motility is considered one of the most useful markers of the quality of spermatozoa in fish (Gallego *et al.* 2018), although there are reports indicating that traditional sperm quality parameter evaluation may not be accurate as an estimate of fertilising ability (Patrizio *et al.* 2008). There is a high correlation between this traditional parameter of sperm quality and fertilisation success in a variety of teleost species (Cabrita *et al.* 2014). In this study we used CASA because the duration of sperm motility in *S. senegalensis* is brief (~1 min) and accurate evaluation of the sperm kinetics is only possible with this kind of system (Gallego *et al.* 2018). Interestingly, the results showed no significant differences between the two groups when total motility was analysed, indicating a homogeneous global motility status between the groups (Fig. 1*a*). This outcome may be unexpected because of the usual assumption that sperm samples from F_1 sole are of poorer quality (Cabrita *et al.* 2006; Valcarce *et al.* 2016). Two important factors that could explain the similar values for sperm quality parameters are the 18-month acclimatisation period for the breeders prior to the experiments and the enriched diet that was provided. Detailed analysis of sperm motion kinetics 30 s after sperm activation revealed slight but significant differences in VCL. A similar, albeit not significant, tendency was found for VSL and VAP (Fig. 1*b*). PI staining showed similar values for wild and F_1 males, around 80% (Fig. 1*a*).

Our results provide the perfect scenario to investigate whether there are differences at a molecular level in the sperm cells that could explain, in part, the differences in reproductive performance reported in this species (Carazo *et al.* 2011), even when general traditional sperm parameters are not markedly altered in F_1 males.

The first molecular parameter analysed was methylation level as an epigenetic mark (Labbé *et al.* 2017). Similar to what was observed in the analysis of traditional parameters, global methylation analysis using the EpiJET DNA Methylation Analysis Kit (*Msp*I/*Hpa*II), which uses the *Msp*I and *Hpa*II restriction enzymes to analyse DNA methylation status at a specific locus, showed no differences between the two groups (Fig. 2*a*). A complete methylation status was observed in all sperm samples analysed, according to what could be expected in a normal transcriptionally inactive sperm cell with highly condensed DNA, as has been reported in other teleost fish (Kurtz *et al.* 2009; Wu *et al.* 2011). In order to check whether the epigenetic pattern could be altered in the brain, we performed the same

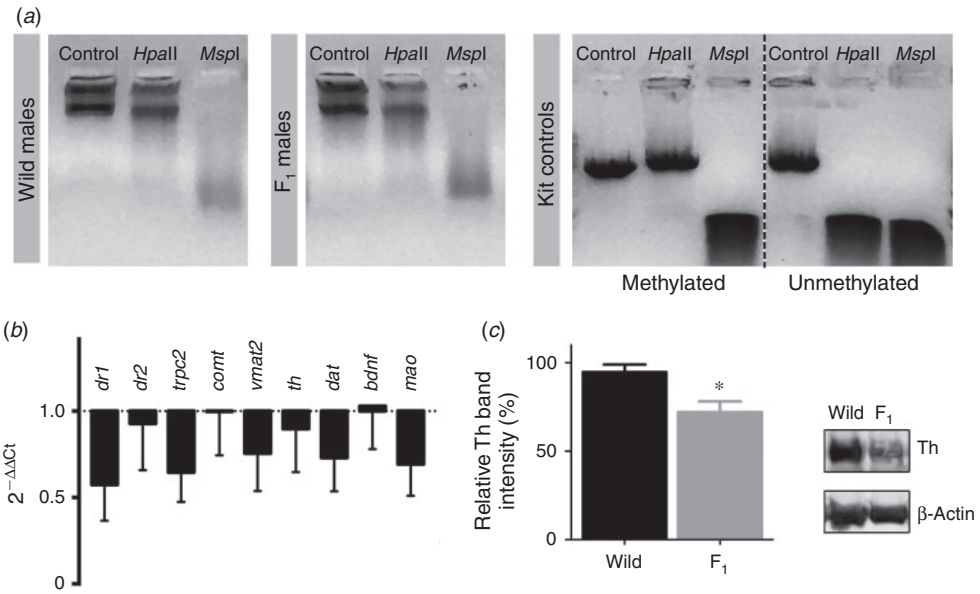

Fig. 3. Molecular analysis of brain tissue. (*a*) Global methylation status of brain sample DNA, determined using an EpiJET DNA Methylation Analysis Kit (Thermo Fisher Scientific) from male Senegalese sole (*Solea senegalensis*) born in a natural environment (wild) and born in captivity (F_1). Three independent experiments of three different pools of three males per group were analysed (data not shown). Unmethylated plasmid DNA (kit control) is digested by both enzymes, whereas methylated plasmid DNA (kit control) is susceptible only to Epi *Msp*I digestion. (*b*) Expression levels of dopamine-related genes relative to the housekeeping gene elongation factor 1 (*ef1*α) were calculated for all samples using the $2^{-\Delta\Delta Ct}$ method. The figure shows expression of each gene in the F_1 group relative to that in wild males, which was set to 1. *dr1*, dopamine receptor 1; *dr2*, dopamine receptor 2; *trcp2*, transducin repeat-containing protein 2; *vmat2*, vesicular monoamine transporter 2; *dat*, dopamine transporter; *mao*, monoamine oxidase; *comt*, catechol-*O*-methyl transferase; *th*, tyrosine hydroxylase; *bdnf*, brain-derived neurotrophic factor. Data are expressed as the mean ± s.e.m. of $2^{-\Delta\Delta Ct}$ values from three independent experiments with three replicates for each. No differences in gene expression were detected between the two groups. (*c*) Mean (± s.e.m.) Th protein expression in brain tissue from wild and F_1 male *S. senegalensis* as determined by western blot analysis. β-Actin was used as a loading control. Th expression was assessed for each experimental group and is expressed as percentage of signal intensity over control. $*P < 0.05$ compared with the wild group. Four different males per group were used in three independent experiments.

assay using samples of brain tissue. There were no significant differences between samples from F_1 and wild males, indicating that there are no changes in global methylation in F_1 males in the cells and tissues studied (Fig. 3*a*).

However, DNA is not the only sperm molecule that is delivered to the embryo; since their discover approximately 30 years ago, sperm-borne RNAs (both large and small and coding and non-coding) have been reported in many organisms (Schuster *et al.* 2016). Recent studies have suggested certain sperm mRNAs as good molecular markers of sperm quality (Ostermeier *et al.* 2002; García-Herrero *et al.* 2011). It is known that although spermatozoa are transcriptionally inactive cells (Grunewald *et al.* 2005), these transcripts are remnants from spermatogenesis and could be relevant in fertilisation and early embryo development (Meseguer *et al.* 2004). Surprisingly, some of these transcripts are preserved from teleosts to human (Guerra *et al.* 2013; Valcarce *et al.* 2013; Robles *et al.* 2017). In the present study we analysed the presence of three of these transcripts successfully validated for *D. rerio* and *Sparus aurata* by our group (Guerra *et al.* 2013; Valcarce *et al.* 2015), namely *bdnf*, *dmrt1* and *fsh*. There were no significant differences in these mRNAs between F_1 and wild males (Fig. 2*b*).

To evaluate possible differences in the dopaminergic pathway between groups, we analysed the expression of key genes in this pathway. Surprisingly, none of the eight transcripts studied (*th*, *dr1*, *dr2*, *trcp2*, *vmat2*, *dat*, *mao* and *comt*) differed significantly between the two groups (Fig. 3*b*). Moreover, we investigated *bdnf* transcript expression in brain because of its critical role in neuron maintenance, neuron survival, plasticity and neurotransmitter regulation (Lima Giacobbo *et al.* 2018) and its link to behaviour in teleost species (Borrelli *et al.* 2016). Again, there was no significant difference between the two groups (Fig. 3*b*). Interestingly, analysis of brain tissue revealed downregulation of the expression of Th protein, a key enzyme for dopamine synthesis, in F_1 males (Fig. 3*c*), thus suggesting a possible modification in the post-transcriptional regulation of protein expression. This is extremely interesting because Th catalyses the conversion of the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) at the beginning of the dopamine biosynthetic

pathway (Daubner *et al.* 2011). Mesolimbic dopaminergic signalling plays a key role in the activation of the reward pathway, either by natural or maladaptative rewards. Therefore, it may be possible that a dysfunction in the reward pathway in the Senegalese sole is responsible for reproductive failure in F_1 males reared in captivity.

A subsequent experiment to evaluate the sperm-specific miRNA population was performed in sperm samples (Fig. 2*c*). Although the regulatory role of miRNAs has only recently started to be studied in the human reproductive system, miRNAs have been shown to play important roles in the control of reproductive functions in male fertility and in early embryo development (Eisenberg *et al.* 2015). Some authors consider miRNAs as suitable candidates for use as biomarkers of male reproduction and fertility because of their stability, and envision that the development of these markers will be of high financial interest to the animal production industry (Pratt and Calcatera 2017). More interestingly, miRNAs could be one of the vehicles for the transmission of specific characteristics to the progeny (Dickson *et al.* 2018). These small non-coding RNAs are highly conserved among animal species, including human, suggesting that they may act as regulators of gene expression across diverse animal species (Pasquinelli *et al.* 2000; Hertel *et al.* 2012). The set of miRNAs evaluated in the present study were *miR-141-3p*, *miR-let7-d* and *miR-200a-5p*. *miR-141-3p* is a mitochondria-related miRNA that is involved in the modulation of ATP production and the induction of oxidative stress (Ji *et al.* 2015). This miRNA has been suggested as a biomarker of infertility in human spermatozoa (Abu-Halima *et al.* 2013). In somatic cells, *miR-200a-5p* is involved in the modulation of necrotic pathways, presumably triggered in response to oxidative stress (Yang *et al.* 2018*a*), whereas in germ cells *miR-200a-5p* is involved in spermatogenesis and sperm function (Abu-Halima *et al.* 2013; Kotaja 2014). *miRlet-7-d* is one of the miRNAs identified in zebrafish spermatozoa (Jia *et al.* 2015). In mammals, *let-7* expression is high during embryogenesis and brain development (Thomson *et al.* 2004, 2006; Schulman *et al.* 2005; Wulczyn *et al.* 2007) and remains high in adult tissues (Sempere *et al.* 2004; Thomson *et al.* 2004). This molecule seems to be an essential regulator of terminal differentiation. During male germ cell development, the expression of the *let7* miRNA family changes (McIver *et al.* 2012), and in mice this molecule is also differentially present throughout epididymal maturation (Nixon *et al.* 2015). Interestingly, there were differences in two of the three miRNAs studied (*miR-let7-d* and *miR-200a-5p*) in sperm samples from the F_1 and wild groups (Fig. 2*c*). The greatest difference between the F_1 and wild groups was seen in *miR-200a-5p* expression, which was approximately twofold higher in the F_1 group. In a recent previous study in zebrafish, the *miR-200* cluster was revealed to play a role regulating sperm motility (Xiong *et al.* 2018). Sperm motility (VCL, VSL and VAP) was significantly improved in knockout fish with deletion of the *miR-200* cluster on chromosome 23. Interestingly, no differences were reported in total motility in the knockout models (Xiong *et al.* 2018). The findings of that study support those of the present study, in which we found differences between F_1 and wild samples only in VCL. These data suggest a potential molecular deregulation in F_1 sperm samples. Although this change seems to have no effect on sperm quality parameters in adult animals, it should be considered as a possible way of transmitting deregulation to the F_2 generation. Recently, it was reported that reduced levels of certain miRs in the spermatozoa of mice and men exposed to early life stress could contribute to transmission of stress phenotypes across generations (Dickson *et al.* 2018); in the same way, changes in certain sperm miRNAs in the sole could contribute to the transmission of reproductive dysfunction across generations. With this in mind, we performed an *in silico* study using TargetScanFish software to find *miR-let7-d* and *miR-200a-5p* targets, which, interestingly, revealed *th* and other mRNAs involved in dopaminergic pathway as targets of *miR-200a-5p*. A recent study in zebrafish reported a role for the miR-200 family in axon growth and target innervation of dopaminergic neurons (Yang *et al.* 2018*b*).

In conclusion, the present study contributes to our knowledge of the reproductive biology of *S. senegalensis* after domestication. Our results point to changes in the Th protein as one important cause of reproductive dysfunction in F_1 males that could be crucial in explaining the absence of courtship behaviour in these individuals, as well as to sperm *miR-let7-d* and *miR-200a-5p* as possible vehicles for the transmission of reproductive dysfunction to the F_2 generation. Further studies should analyse miR-200 and Th protein levels in the brains of wild-born males and males born in captivity to provide more evidence regarding the role of the miR-200a-5p/Th pathway in reproductive capacity.

Conflicts of interest

The authors declare no conflicts of interest.

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