



Reproductive ethogram and mate selection in captive wild Senegalese sole (*Solea senegalensis*)

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Abstract

Senegalese sole (*Solea senegalensis*) have a high potential for aquaculture that is hampered by reproductive behavioural problems. These problems result in limited breeder participation in spawning. The present study provided an ethogram and described mate selection and spawning of captive wild Senegalese sole. Two tanks of breeders were studied that had 29 and 25 breeders (mean weight = 1.6 ± 0.1 kg). The behaviour was studied during 20 periods of 24 hours: 10 periods where spawning events were recorded and 10 control periods without spawning events. Periods where spawning occurred had three times more locomotor activity than periods without spawning. Two distinct behaviours, termed the “following” behaviour and the “coupled swim”, were only observed during periods with spawning. The courtship sequence (n=12) began with males predominantly involved in “following” behaviours, whilst females remained mainly stationary on the bottom of the tank. Males rested on the females and encouraged the females to begin swimming. When the female began to swim the male swam under the female and the pair made a “coupled swim” to the surface to release gametes. Gamete release was strictly in pairs of one male with one female. Failed “coupled swims” without gamete release were 5.6 times more frequent than successful “coupled swims”. Mate selection was evident as the sole engaged in: paired spawning, males displayed to females, males encouraged females to spawn and females accepted or rejected the male’s advances. The mate selection process provided the opportunity for fish to dominate the spawning and also demonstrated how fish were excluded from spawning.

Additional key words: behaviour; flatfish; courtship; spawning; paired spawning; following.

Abbreviations used: DVR (Digital Video Recorder); EOG (electro-olfactogram); FCR (food conversion ratio); FEDER (Fondo Europeo de Desarrollo Regional); FELASA (Federation of Laboratory Animal Science Associations); IEO (Instituto Español de Oceanografía); INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentarias); IRTA (Institut de Recerca I Tecnologia Agroalimentaria); JACUMAR (Junta Nacional Asesora de Cultivos Marinos).

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Introduction

Spawning behaviour, courtship, mate selection and gamete release can determine which individuals achieve reproductive success through the coordinated release of gametes to produce viable eggs and off-spring. A wide range of spawning behaviours have been described (see reviews by Balon, 1975; Berglund, 1997) amongst spe-

cies and also within some species. Mate selection and gamete release is a key factor that determines which genes or phenotypes are either passed to the next generation or lost (Berglund, 1997; Edward, 2015). Therefore, the description and understanding of a species spawning behaviour has implications for evolution and practical applications for aquaculture and conservation biology to maintain genetic pools and improve stocks.

Flatfish spawning behaviour has been described to varying degrees for nine species, from four families, which are four Bothidea species: kobe flounder (*Crossorhombus kobensis*) (Moyer *et al.*, 1985), eyed flounder (*Bothus ocellatus*) (Konstatinou & Shen, 1995), large scale flounder (*Engyprosopon grandisquama*) (Manabe *et al.*, 2000) and wide-eyed flounder (*Bothus podas*) (Carvalho *et al.*, 2003); three Pleuronectidae species: winter flounder (*Pseudopleuronectes americanus*) (Breder, 1922; Stoner *et al.*, 1999), European plaice (*Pleuronectes platessa*) (Forster, 1953), greenback flounder (*Rhombosolea tapirina*) (Pankhurst & Fitzgibbon, 2006); one Soleidae, common sole (*Solea solea*) (Baynes *et al.*, 1994) and one Paralichthyidae species, bastard halibut (*Tarphops oligolepis*) (Manabe & Shinomiya, 2001). The act of gamete release described for eight of these species was strictly paired spawning when a single male released gametes with a single female. The winter flounder was the exception and was described to release gametes in both a pair and a group (Breder, 1922; Stoner *et al.*, 1999). The gamete release in a group consisted of a single female spawning with two or more males, which is a common spawning behaviour in round fish species (Domeier & Colin, 1997; Ibarra-Zatarain & Duncan, 2015). The eight species that spawned as a pair formed a pair on the bottom before making an upward synchronised coupled swim to release gametes. During the upward synchronised swim the pair held the dorsal surface of one fish close to or pressed against the ventral surface of the second fish so that the genital pores of the two fish were held together. Gametes were released at the peak of the ascent and the fish then returned to the bottom. The winter flounder also swam upwards, but in a spiral to release gametes that were mixed by the circular swimming (Stoner *et al.*, 1999).

The act of gamete release or spawning is preceded by the courtship and mate selection behaviour. Descriptions of courtship and mate selection were reported to varying degrees for eight of the above species. Generally, during the courtship the female flatfish were approached by males and the female either accepted the advance to spawn or swam away rapidly. The males were more active than females (Baynes *et al.*, 1994; Stoner *et al.*, 1999) and played a more active role in the courtship by displaying to the largely stationary female (Moyer *et al.*, 1985; Manabe *et al.*, 2000; Manabe & Shinomiya, 2001; Carvalho *et al.*, 2003; Pankhurst & Fitzgibbon, 2006). The behaviours of the male display included raising the head, arching the body, waving the pectoral fin, short upward swims, and touching or resting on the female (Moyer *et al.*, 1985; Manabe *et al.*, 2000; Manabe & Shinomiya, 2001; Carvalho *et al.*, 2003; Pankhurst & Fitzgibbon, 2006). The males would display in front of a single female

and if the female accepted the pair would begin the synchronised coupled swim to spawn. A different behaviour that appeared to be part of the display and courtship of the winter flounder was described as a “following” behaviour (Stoner *et al.*, 1999). In this “following” behaviour the males and females swam following each other in a kind of procession. This “following” behaviour was associated with spawning and was uncommon outside of the spawning period. An important aspect of the courtship in the four Bothidea species (Moyer *et al.*, 1985; Konstatinou & Shen, 1995; Manabe *et al.*, 2000; Carvalho *et al.*, 2003) and the bastard halibut (Manabe & Shinomiya, 2001) was that the males defended a territory that included a harem of females or females visited the defend territory for courtship and spawning. These five species were studied in the natural environment. As a whole these studies indicate a mating system that is common in the animal kingdom where males compete with each other to gain access to females that then accept or reject the male’s approaches (Reynolds, 1996).

Senegalese sole (*Solea senegalensis*) have excellent aquaculture potential. This potential has attracted industrial investment during the global economic crisis (2008-2015). This investment has increased world production of Senegalese sole from 60 tons in 2008 to 1065 tons in 2014 (FAO, 2011-2016). During the same period (2008-2012) aquaculture production of the European industry as a whole has stagnated and declined. Attractive aspects for the culture of Senegalese sole include: larval culture that is uncomplicated with commercially viable survivals (Howell, *et al.*, 2011; Morais *et al.*, 2014), juvenile growth (450 g in 14 months) that is similar or better than other southern European aquaculture species such as gilthead seabream (*Sparus auratus*) (Anguis & Cañavate, 2005) and perhaps most importantly a high market price for whole fish that exceeds production costs (Morais *et al.*, 2014).

However, Senegalese sole culture does face bottlenecks that need to be addressed to facilitate and continue this increase in production. Producers reported that the principal production problems were spawning of hatchery reared stock, control of diseases and feed-related problems such as poor growth and food conversion ratios (FCRs) (Howell *et al.*, 2011; Morais *et al.*, 2014). Producers cannot produce eggs from hatchery reared stocks and must use wild breeders. Wild stocks held in captivity spawn eggs of sufficient quantity and quality to form the basis of commercial production (Anguis & Cañavate, 2005; Martín *et al.*, 2014). However, wild Senegalese sole breeders appear to exhibit dominance in the spawning tanks as microsatellite analysis of paternity has demonstrated that 8.7% to 51.7% of breeders actually participated in spawning

(Porta *et al.*, 2006b; Martin *et al.*, 2014) and that fidelity of spawning pairs appeared to exist (Martin *et al.*, 2014). This low participation of breeders in spawning means the effective breeding population was smaller than the actual size of the broodstock. As a consequence, Porta *et al.* (2006a,b) reported that few families were produced and consequently there was a high loss of genetic variability over one generation from the wild breeders to the offspring. However, the most serious problem is the total failure of hatchery reared stock to produce fertilised eggs. This failure was demonstrated to be due to a behavioural reproductive dysfunction predominantly associated to hatchery reared males (Carazo, 2012), however, to date no peer reviewed studies have been published on the reproductive behaviour of Senegalese sole.

The main aim of this study was to describe in detail the reproductive behaviour of Senegalese sole from the beginning of the courtship to the end of the spawning. Subsidiary aims were to provide (a) evidence to explain the pattern of mating that has been described with microsatellites in captive wild stocks and (b) a reproductive ethogram for the quantitative analysis of Senegalese sole behaviour. This ethogram would represent an important tool to compare the reproductive behaviour between different groups and allow future studies to explain problems associated with reproduction that have been observed in the Senegalese sole.

Materials and methods

All the experimentation on fish that formed part of this study were in agreement with the Spanish and European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA) and approved by the Animal Ethics Committee of IRTA.

Fish and holding conditions

The wild Senegalese sole used in the study were held in the research facilities of the Spanish Institute of Oceanography (Instituto Español de Oceanografía, IEO), Santander. Martin *et al.* (2014) reported the egg production and parental contribution for the same tanks used in the present study. The fish were collected from estuary lagoons close to Huelva, Spain by fishermen using traps. Prior to the study the fish were maintained for six-eight years in the IEO Santander in two rectangular tanks of 14 m³ (2.3 × 6 m). These two tanks of breeders were studied: Tank B2 had 25 breeders (12 females and 13 males) that had a mean weight of 1.6 ± 0.1 kg and the tank biomass of 38.9 kg (2.8 kg/m²).

Tank B3 had a total number of 29 breeders (14 females and 15 males) that had a mean weight of 1.6 ± 0.1 kg and the biomass of fish in the tank was 45.0 kg (3.2 kg/m²). The two groups of breeders were held in identical conditions, with an open flow through water exchange of 300% per day. The fish were fed 1% of the total biomass six days a week (Monday to Saturday) at 11:00 (approximately). The diet was frozen natural food items, mussels, sliced squid and polychaetes. Tanks were in an enclosed building without windows and each tank was covered with shade netting. Photoperiod and temperature were controlled. Photoperiod during the study and the preceding 6-8 years was LD16:8 (Light:Dark) and the day period was from 08:00 to 24:00. Daytime illumination was provided with fluorescent lighting (50 lux at surface). Night illumination was low intensity red light from fluorescent lights covered with a red filter that only permitted the passage of light >720nm wavelength (supergel Rosco, Rosco Iberica S.A. Madrid, Spain www.rosco.com). The red light was adjusted to approximately 5 lux at the water's surface, which enabled recording and observation of the fish behaviour. This illumination system was previously tested and shown to not significantly affect behaviour, locomotor activity or plasma melatonin (Carazo *et al.*, 2013). Temperature during the study period followed a weekly cycle that had been shown to stimulate spawning (Martin *et al.*, 2014). The cycle was Monday to Thursday at 16 ± 1°C and Thursday to Monday at 18 ± 1°C with a period of approximately 24 hours to change between programmed temperatures. During the four years prior to the study, both groups had spontaneous spawning that was stimulated by the cyclic variation in temperature. Each tank had an egg collector that collected eggs from a surface water outlet. The egg collector was checked for eggs each morning at 08:00. Spawning eggs were collected and the following parameters were determined: volume of floating and sinking eggs, stage of development and percentage fertilisation of floating eggs. Martin *et al.* (2014) reported that a total of 8 males and 8 females in tank B3 and 6 males and 4 females in tank B2 participated in the spawning during the year that behavioural studies were made. In tank B3 the spawning was dominated by three males and three females that spawned 85.2% of the eggs by volume. In tank B2 four males and three females spawned 88% of the eggs and one pair in this group spawned 60% of the eggs.

Analysis of behaviour

Digital cameras (Square black and white CCD camera, model F60B/N80-50G, KT&C Co. Ltd., Korea Technol-

ogy and Communications Korea, supplied in waterproof housing by Praesentis S.L. Barcelona, Spain) were used to film the fish behaviour. The cameras were connected to a digital video recorder (model DVR-Camtronics-UCDI-DV4150-1500, supplied by Praesentis S.L.) and 24 h recordings were made during the period of study. Four cameras that had an angle of view of 150% (in air) were installed in each tank. The cameras were positioned just below the water surface angled downwards, at intervals of 1.5 m along the length of the tank. The camera positions enabled 96 % of the entire water column of the tank to be filmed and recorded. Tank B3 was studied from 7 to 23 May and tank B2 from 1 to 17 April.

The behaviour was analysed during different periods of 24 hours that began and ended when the egg collectors were checked at 08:00. The behaviour was analysed during 20 periods of 24 hours, which included 10 periods where spawning events occurred (that will be referred to as periods with spawning) and 10 periods without spawning events (that will be referred to as periods without spawning). The 24-h periods without spawning were randomly selected from each of the two tanks of breeders (5 periods per tank). The behaviours observed during these periods were used to write an ethogram. The ethogram was used to make a general qualitative or subjective description of the courtship and gamete release. Gamete release was visible in the videos as an opaque cloud in the water column. The swollen ovaries of the females were visible in the videos and this characteristic was used to differentiate between males and females. Focal animal observations (Altman, 1974) of sole that engaged in gamete release were made by observing the courtship in both forward and reverse. All behaviours of the sole that released gametes were registered to build a sequence of behaviours before each spawning event. A quantitative analysis of the behaviour was made by counting activity and the behaviours described in the ethogram. The locomotor activity was quantified by scoring each time a sole swam over a line drawn across the middle of the field of vision of each camera as described and validated by Carazo *et al.* (2013). Different behaviours (as described in the ethogram) were counted during the peak half hour (19:45 to 20:15) of locomotor activity. The behaviours were counted by first dividing the peak half hour into 3-min sections. Secondly, the behaviour of each animal on each 3-min time point was registered. The same half hour period (19:45 to 20:15) was analysed for all 10 periods with spawning and the 10 random periods without spawning.

Statistical analysis

All means were presented with standard error (\pm SE). Data were analysed with the Kolmogorov-Smirnov test

and found to have a normal distribution. Profiles of behaviours during different periods were compared with the chi-square test ($p < 0.05$). The analysis of the activity was made with a description of daily activity profiles (Bayarri *et al.*, 2004). The program Sigma Stat (Systat Software Inc., Germany) was used for statistical analysis.

Results

Spawns collected

During the period of recording, eggs were collected from tank B3 on six different days. The average volume of eggs collected was 307.9 ± 174.7 mL, which included eggs that did not float (unviable) and floating eggs (viable). The floating eggs had an average fertilization of 91.4 ± 23.5 %. The eggs on two of the collection days had different stages of development, which were approximately 3 to 5 hours apart and this indicated that there were two different spawns during the spawning period prior to egg collection. This was confirmed as a total of eight successful courtships were observed in the six periods prior to the collection of a spawn. Viable eggs were collected from tank B2 on four different days. The average volume collected was 187.9 ± 147.5 mL and floating eggs had 87.0 ± 9.1 % average fertilization. Successful courtships were observed during the four periods prior to collecting fertilised eggs. Unviable (non-floating) eggs with no fertilisation were collected on three days. During the period prior to the collection of unviable eggs, no successful courtship was observed.

Locomotor activity

The locomotor activity of the Senegalese sole breeders had a circadian rhythm with consistent patterns in the level of activity during the 24-hour period (Fig. 1). The mean activity during the 24-hour periods with spawning (9.7 ± 10.9 movements/hour) was higher than during periods without spawning (4.5 ± 3.1 movements/hour). During periods with spawning the activity began to increase during the afternoon and activity was higher than the mean from 15:00 to 23:00 with a peak period during the hours 17:00 to 21:00. The peak half hour of activity was 19:45 to 20:15. Activity during periods without spawning was 3 times less during the peak half hour of activity and the length of the period of increased activity above the mean was 2 hours shorter (19:00 to 01:00). All activity profiles had a small and similar increase in activity at 11:00, which was the hour of feeding.

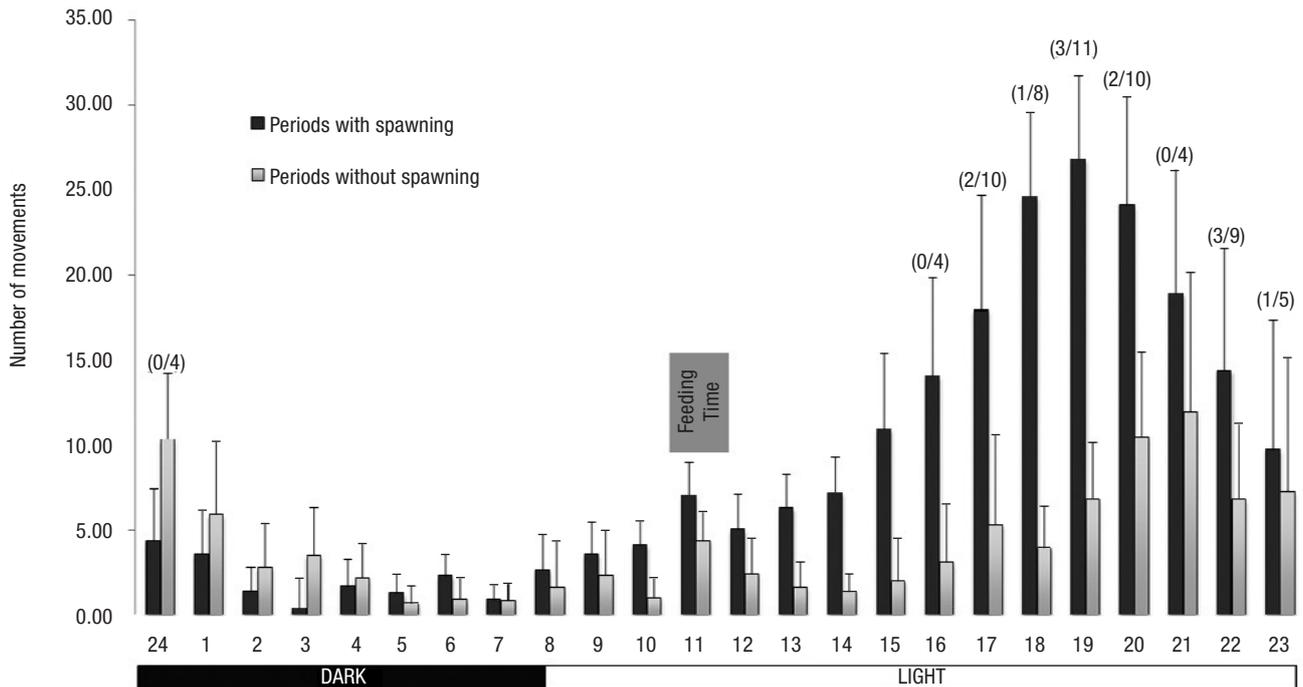


Figure 1. Mean locomotor activity (number of times a sole crossed a line in the field of view of each camera) of the captive wild Senegalese sole (*Solea senegalensis*) breeders during the 24-hour periods that included spawning (n=10) and periods without spawning (n=10). Bar at the bottom: dark indicates night with 5 lux red light and white indicates day with 50 lux white light. The numbers in brackets indicate number of synchronised coupled swims with gamete release / number of failed synchronised coupled swims.

Ethogram

The behaviour observed was broken down into isolated behaviours or actions and described in the form of an ethogram. The behaviours were entered in four broad groups termed, “stay quiet”, “rest head”, “burying” and “swimming” (Table 1).

Stay quiet behaviour: Senegalese sole breeders were observed to be generally inactive and for much of the time remained still and quiet on the bottom of the tank without any activity (Fig. 2; Table 1). The captive Senegalese sole had a daily inactivity rate of 70%, with differences between males and females. The swollen ovaries of the females were visible in the videos and this characteristic was used to differentiate between males and females (Fig. 2; videos S1, S2 and S3 [suppl.]). During the full 24-hour period, males exhibited activity on average every 1.7 ± 1.3 min while females moved every 4.3 ± 1.9 min. This stationary, still and quiet behaviour was termed “stay quiet”. The “stay quiet” behaviour was divided into two types, (a) “stay quiet” without interactions when an animal was alone and (b) “stay quiet” with actions when the animal stays quiet, but has interactions from another animal, which is often resting on the “stay quiet” animal. During the

peak half hour of activity (Fig. 1) $40.1 \pm 3.9\%$ of the behaviours were “stay quiet” during periods with spawning and $61.7 \pm 4.3\%$ of the behaviours were “stay quiet” during periods without spawning. The “stay quiet with interactions” behaviours were similar $10.3 \pm 0.9\%$ and $9.3 \pm 1.8\%$ respectively between periods with and without spawning (Fig. 3).

Rest head behaviour: A behaviour that was frequently observed was when an individual rested its head on another fish. This behaviour was termed “rest head”. The “rest head” behaviour was also classified by where the head was rested on the body of the lower fish, which was either on the head, back, ovary or tail (Fig. 2; Table 1; videos S1 and S2 [suppl.]). The fish engaged in “rest head” behaviours were observed to remain still in one position or move over the fish resting the head on different parts of the lower fish. A particular behaviour that was observed in relation to the “rest head” behaviour was termed the “guardian” behaviour. The “guardian” behaviour was a situation when a fish actively guarded another fish from the advances of a third fish. The “guardian” behaviour was often observed when a second male approached a couple where the male was resting its head on the female. The male resting on the female would often place its body between the second male and

¹ Videos lost considerable quality due to format changes and editing compared to the original videos viewed and analysed on the recorder.

Table 1. Ethogram of principal behaviours observed in association with gamete release in wild Senegalese sole (*Solea senegalensis*) breeders. The behaviours were organised in four broad behavioural groups, stay quiet, rest head, burying and swimming.

Behaviour group	Behaviour / action	Description
Stay quiet	Stay quiet solo	A single fish staying quiet on the bottom of the tank
	Stay quiet actions	A fish staying quiet with another fish touching with or without actions
Rest head	Rest head - Head	A fish resting the head on the head of another fish
	Rest head - Back	A fish resting the head on the back of another fish
	Rest head - Ovary	A fish resting the head on the ovary of another fish
	Rest head - Tail	A fish resting the head on the tail of another fish
	Guardian	A fish rests the head on another fish and actively guards the fish from the advances of a third fish.
	Whole fish	A fish uses the whole body to cover the whole body of another fish
Burying	Burying solo	A series of waves of the body and lateral fins starting from the head by raising the head that in sand would bury the fish (Gibson, 2005)
	Burying under	The burying behaviour of a fish under another fish. Appears to be an action to reject the fish on top.
	Burying on top	The burying behaviour of a fish on top of another fish. Appears to be an action to make the fish on the bottom swim.
Swimming	Swim solo	A fish swimming solo without interactions with other fish.
	Swim to	A swim to get closer to another fish. Usually short and may finish in rest head, but can be the final part of a long swim.
	Swim away	A swim away from a fish, after the initial swim away the swim may be a long distance. Females use this to avoid advances from a male.
	Followed	A fish at the head of a procession of swimmers, one or more. This behaviour can last for a long period. The following fish copy nearly exactly the manoeuvres of the lead “followed” fish.
	Follow	A fish following the lead “followed” fish. The following fish copy nearly exactly the manoeuvres of the lead “followed” fish. This behaviour can last for a long period.
	Coupled	The coupled swim by a male and female towards the surface to release gametes. The coupled swim can be broken before gamete release

female and cover the eyes of the female. The “guardian” male was also occasionally observed to push the second male away with its tail. Lastly, a behaviour where the upper fish was observed to completely cover the lower fish was termed the “whole fish” behaviour. During the peak half hour of activity (Fig. 1) $10.3 \pm 1.0\%$ (periods with spawn) and $9.6 \pm 0.9\%$ (periods without spawn) of the observed behaviours were the “rest head” behaviours (Fig. 3).

Burying behaviour: The sole have a behaviour in which the fish make a series of waves of the entire body including the dorsal and anal fins. The wave started from the head by raising the head and then travelled to the tail. This behaviour in sand would move the sand to bury the fish (Table 1). This behaviour was termed the “burying” behaviour and has been described for common sole (Kruuk, 1963) and flatfish in general (Gibson, 2005). The “burying” behaviour was observed in three different situations. The sole were observed to make the “burying” behaviour when solo or isolated, when underneath another fish or when on top of another fish. The “burying” behaviour when the fish was solo was termed “burying solo”. The “burying” behav-

our when the fish was under another fish was termed “burying under”. The “burying under” behaviour was observed to disturb the upper fish in order to give a reaction such that the upper fish swam away. The “burying” behaviour when the fish was on top of another fish was termed “burying on top”. The “burying on top” behaviour was observed to disturb the lower fish that would swim away and this often gave a male fish the opportunity to swim under a female fish.

Swimming behaviour: The behaviour to move position, which as described in other flatfish was achieved with rhythmic waves of the body and fins (dorsal, anal and caudal). Once moving swimming could also be achieved by gliding. In general terms, swimming was more common along the bottom without losing contact with the bottom. However, swimming in the water column was also frequently observed. Swim behaviours were divided into “swim solo” (Table 1) and “swim with interactions” when two or more swimming fish interacted. The “swim with interactions” behaviours were divided into, “swim to”, “swim away”, “followed”, “follow” and “coupled” (Table 1). The “swim to” and “swim away” behaviours indicated that a fish

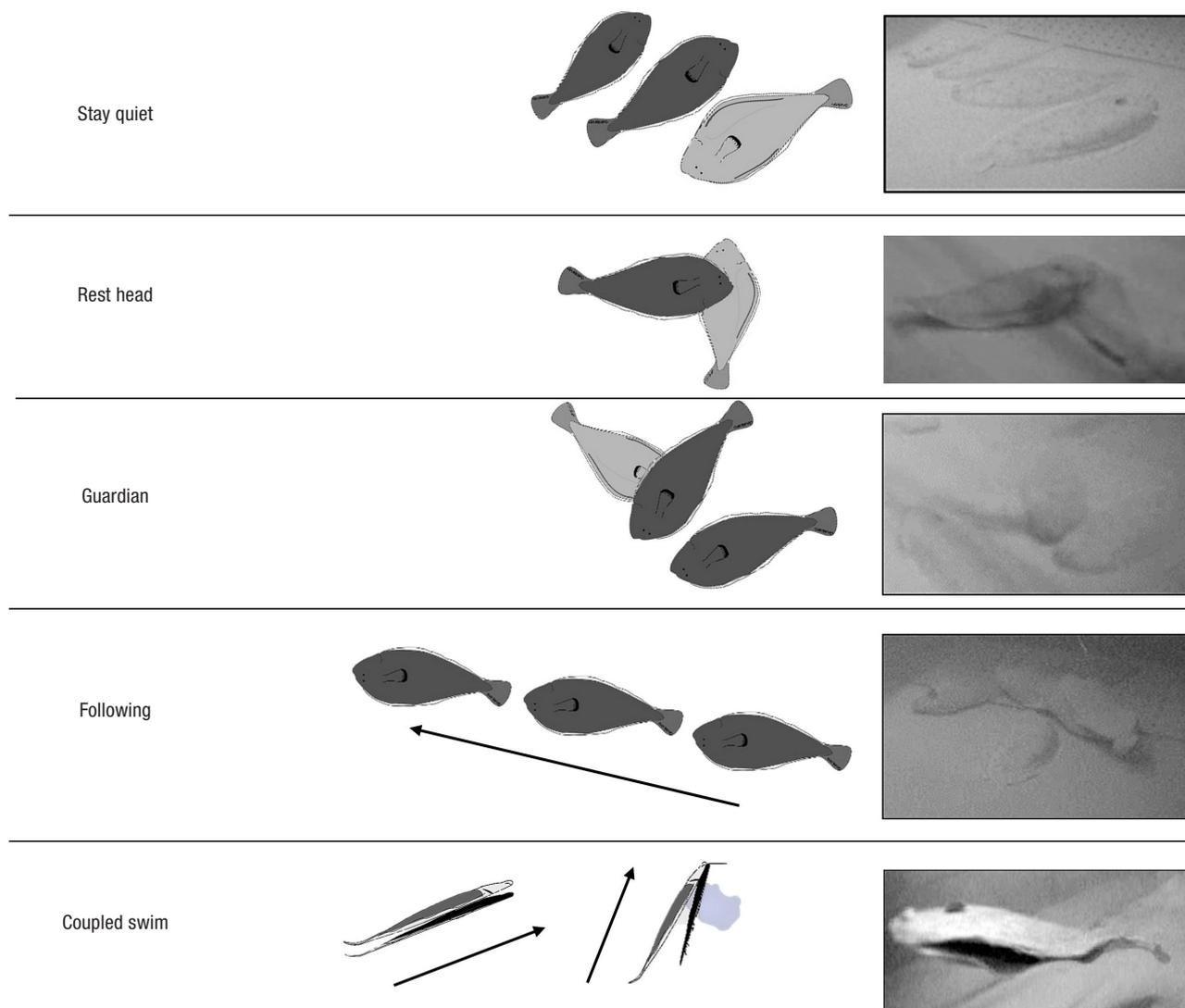


Figure 2. Video captures and diagrams of different behaviours associated with gamete release in captive wild Senegalese sole (*Solea senegalensis*) breeders. Males are represented as dark sole and females as lighter sole.

was reacting to another fish either swimming towards a second fish or swimming away from a fish. The “swim to” or “swim away” behaviours were often the initiation or finalisation of a longer “swim solo”. A distinct behaviour that was observed was when two or more fish swam in a procession where the fish that followed a lead fish copied closely the swimming of the lead fish. These distinct behaviours were termed in general “following” behaviours and to identify the position in the procession the behaviour of the lead fish were termed “followed” behaviour and fish that followed the lead fish were termed “follow” behaviour (Table 1; video S3 [suppl.]). Lastly “coupled swim” was when a female and male pair swam in synchrony. The male was always under the female pushing or holding its upper side against the lower side of the female to hold the genital pores in close proximity. The coupled swim began on the bottom and if uninterrupted

finished at the surface with gamete release. Gamete release was visible in the videos as an opaque cloud in the water column (video S2 [suppl.]). During the peak half hour of activity (Fig. 1) in periods with spawning 38.1% of behaviours (swim solo + swim follow + swim followed) were swimming behaviours, which was over twice the $17.9 \pm 3.8\%$ of swimming behaviours observed in periods without spawning (Fig. 3). The “following” swimming behaviours were only observed in periods with spawning. The “coupled” swimming and failed “coupled” swimming behaviours were also only observed in periods with spawning.

Courtship and spawning

The courtship and spawning was divided into three periods or steps, starting from an initial or baseline,

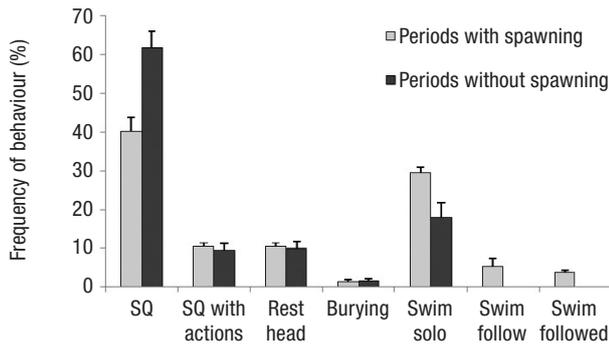


Figure 3. Mean frequency (% of total) of behaviours of captive wild Senegalese sole (*Solea senegalensis*) breeders during the peak half hour of activity (19:45 to 20:15) in periods with spawning (n=10) and periods without spawning (n=10). SQ = stay quiet.

Step 0 with no behaviours that appeared to be related with the courtship, followed by the periods that can be briefly described as: 1) A period of intense activity between a potential female spawner, males and amongst males; 2) the male rests on the female making different behaviours until the female swam from the bottom of the tank; 3) the paired female and male made a “coupled” swim in synchrony to the water’s surface. At the surface the pair held the genital ducts closely together and released the gametes.

Step 1: The activity in the tank increased (Fig. 4). However, the increase in activity was attributed predominantly to males. The males participated in the behaviours “swim followed” and “swim follow”, where the fish swim in a procession around the tank. Each fish in the procession closely followed the behaviours of the lead fish. The “following” behaviour was a kind of parade, chase or persecution; however, there was no sign of aggression or competition. The “following” swimming behaviours involved many different fish (mainly males) and used the entire tank to on occasions swim the entire length of the tank and enter and turn in the corners of the tank. The “following” behaviours were also performed by the females, but to a lesser extent. The females principally remained “stay quiet” on the bottom with principal movements being short swims to reposition often in relation to contacts with other fish. The males were observed to centre attention resting on the females and using all the “rest head” behaviours. The male would constantly change position and “rest head” behaviours. Both males and females were also observed to use the “burying” behaviours: “burying under” and “burying on top”. The females used the “burying under” behaviour to reject the “rest head” behaviours of the male. The male used “burying on top” behaviour to encourage the female to swim. The “rest head” and “burying” behaviours continued until the female began to swim. The female would often

swim to relocate to a different position and the fish would again begin “following” behaviours. Step 1 was a prolonged period with relatively high activity of 2-3 times the activity normally observed and that lasted for a period of 1-2 hours (Fig. 4). The fish that participated in gamete release were observed to also participate in “following” behaviours before 10 of the 12 gametes releases that were observed (Table 2). The “following” behaviours were only observed in periods preceding the collection of a successful spawn and was not observed during the half hour of peak activity in periods without spawning (Fig. 3).

Step 2: As indicated in Step 1, the behaviours of “rest head” of the male on the female often resulted in the female swimming from the bottom (videos S1 and S2 [suppl.]). Step 2 was characterised with this sequence of behaviours that resulted in the female swimming and the male immediately placing itself under the female to begin a “coupled” swim. The “coupled” swim either continued with a rise through the water column or was broken soon after initiation with the two fish separating. This attempted “coupled” swim when the fish broke apart was considered a courtship failure type 1. Step 2 would last 10-15 min (Fig. 4). Rest the head actions were observed prior to all coupled swims, the most common being “rest head–tail” and the least common “whole body” (Table 2). However, rest the head actions were common behaviours that were also observed outside of the courtship and the frequency of the “rest head” behaviours was similar in periods with and without spawns (Fig. 3).

Step 3: The “coupled” swim continued to the surface and gametes were released (video S2 [suppl.]). The entire swim lasted 1-3 min and gamete release was 5-10 sec from when gametes were first visible until the couple broke apart. This behaviour was observed without variation for all spawns. Successful “coupled” swims with gamete release were observed between 17:00 and 23:00 (Fig. 1). Successful coupled swims were most common with the peak of activity with 8 spawns between 17.00 and 20:00. However, the “coupled” swim was also observed to be interrupted before gamete release in a courtship failure. The principal causes that interrupted the “coupled” swims were divided into three types; type 1 as indicated above in Step 2 the female avoids the males attempt to swim under and almost immediately brakes the “coupled” swim; type 2, a second male disrupted the swim; type 3 the “coupled” swim collided with the tank wall or an airline and the couple broke apart. The three types of courtship failure were common in a period with spawning and were observed from 16:00 to 24:00 (Fig. 1). Courtship failures were also most common during the peak of activity with 39 failures between 17:00 and 20:00.

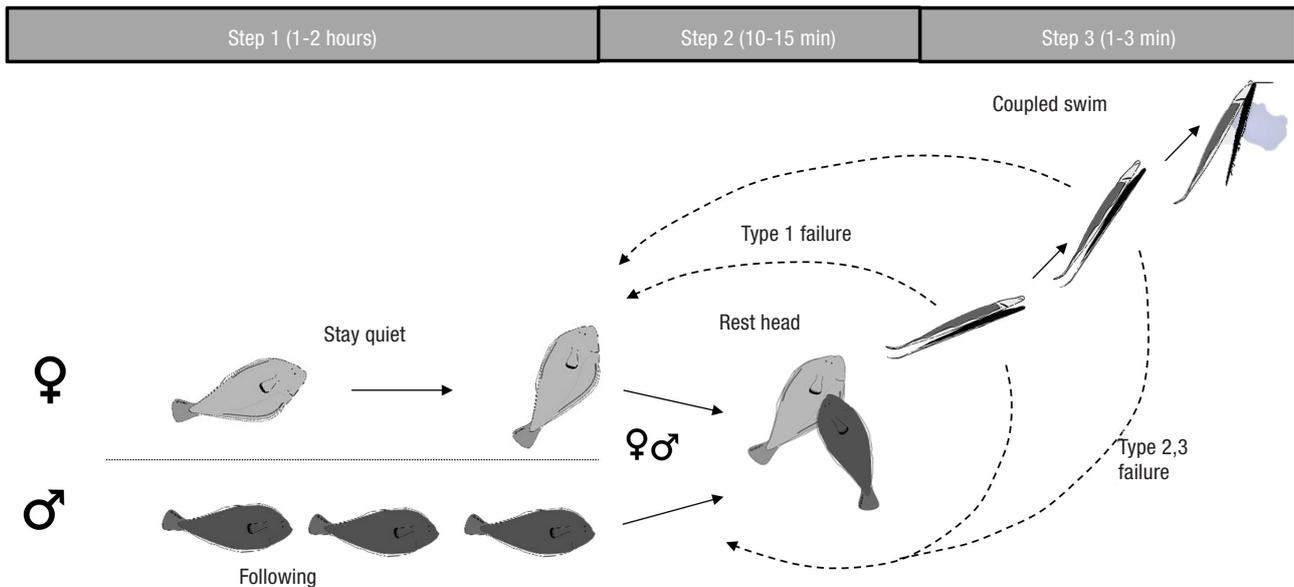


Figure 4. The three steps in the courtship, from mate selection through to gamete release of captive wild Senegalese sole (*Solea senegalensis*) breeders. Times at the top indicate the approximate time period of each step. The predominant behaviours associated with each step are indicated. Dashed lines indicated when failed coupled swims took place: type 1 was the female swam away at the initiation of the coupled swim; and type 2 and 3 was when the couple was broken apart by interference from a second male (type 2) or a collision with the tank wall or other object (type 3).

Failed spawning attempts (total 67) were 5.6 times more frequent than successful spawns (12) over the 10 periods with spawning.

Discussion

This is the first description of courtship and spawning behaviour for the Senegalese sole. The courtship and spawning was similar to other flatfish species and was divided into three steps. The time at which successful spawns were observed was from 17:00 to 24:00 with 8 of the 12 spawns observed between 17.00 and 20:00. This places the time of all spawns later in the “day” before the lights were switched off at 24:00. The time of spawning of other flatfish species was variable, but usually during or close to darkness or the night. Eyed flounder (Konstantinou & Shen, 1995), Largescale flounder (Manabe *et al.*, 2000) and bastard halibut (Manabe & Shinomiya, 2001) spawned at dusk. Wide-eyed flounder (Carvalho *et al.*, 2003) spawned at dawn. Winter flounder (Stoner *et al.*, 1999), greenback flounder (Pankhurst & Fitzgibbon, 2006) and common sole (Baynes *et al.*, 1994) spawned during the night. Kobe flounder (Moyer *et al.*, 1985) spawned during the day in association with the high tide. Common sole (through video recordings; Baynes *et al.*, 1994) and Senegalese sole (through egg collection; Oliveira *et al.*, 2009) were observed to spawn in the first part of the night, which was actually at a similar time (19:00-

23:00) as the Senegalese sole in the present study. This observation of Senegalese sole spawning at the same time, but under different light conditions early night (Oliveira *et al.*, 2009) or late day (present study) has various possible explanations. In the present study the sole did not receive any natural light and received a low intensity (50 lux) constant photoperiod (LD8:16) that possibly did not strongly entrain the biological rhythm. Under these conditions the rhythm that controls gamete release could maintain a spawning time similar to the natural spawning time despite of the different photoperiod. This situation has been described for reproductive development (Bromage *et al.*, 2001) and growth (Duncan *et al.*, 2002). Alternatively, the spawning period in the absence of a strong photoperiod signal may have entrained to other stimulus such as disturbances during the working day, which was from 08:00-17:00. The spawning time being associated to the period without disturbances after 17:00. Lastly it cannot be discounted that the natural spawning time is close to dusk extending from before to after darkness is initiated.

The act of gamete release (Step 3) was only observed between a pair, one male with one female. This gamete release only as a pair was also found in eight of the nine flatfish species that have been observed spawning. These eight species were kobe flounder (Moyer *et al.*, 1985), eyed flounder (Konstantinou & Shen, 1995), largescale flounder (Manabe *et al.*, 2000), wide-eyed flounder (Carvalho *et al.*, 2003), European plaice (For-

Table 2. Behaviours observed by the male and female captive wild Senegalese sole (*Solea senegalensis*) breeders proceeding each gamete release (total n = 12 gamete releases).

Step	Behaviour	Tank B2 n = 4	Tank B3 n = 8	Combined n = 12
1	Following	4	6	10
1-2	Rest head - Back	2	6	8
1-2	Rest head - Ovary	2	5	7
1-2	Rest head - Tail	4	7	11
1-2	Guardian	1	4	5
2-3	Coupled swim	4	8	12
3	Gamete release	4	8	12

ster, 1953), greenback flounder (Pankhurst & Fitzgibbon, 2006), common sole (*Solea solea*) (Baynes *et al.*, 1994) and bastard halibut (Manabe & Shinomiya, 2001). The exception was winter flounder that were observed to also spawn as groups of one female and more than one male (Stoner *et al.*, 1999). On occasions in the present study other male Senegalese sole were observed to swim with the pair that was making the synchronised coupled swim. However, these males did not participate in gamete release and either had no specific interaction or the interaction of the second male caused the pair to break the coupled swim to give a failed spawning attempt (failed spawn, type 2). It would appear that spawning in groups is uncommon in flatfish compared to round fish. Spawning in groups of one female with two or more males is common in round fish (Domeier & Colin, 1997) such as gilthead seabream (*Sparus aurata*) (Ibarra-Zatarain & Duncan, 2015). As was observed in the other eight flatfish species that spawned in pairs the Senegalese sole formed the pair on the bottom and swam up through the water column in synchrony. The synchronised coupled swim of Senegalese sole (Step 3) was the same as that described for common sole (Baynes *et al.*, 1994). However, the behaviour at the initiation (Step 2) of the synchronised spawn was slightly different. As in other species such as common sole (Baynes *et al.*, 1994) and winter flounder (Stoner *et al.*, 1999), the Senegalese sole females moved less and remained mainly static while the male moved to initiate the synchronised coupled swim. The male common sole was described to approach the female from behind and quickly worked its way under the female (Baynes *et al.*, 1994) compared to the Senegalese sole male that would rest its head on the female and move quickly over the female, protecting her from other males and appeared to “annoy” the female to elicit that the female swam from the tank bottom. These behaviours to elicit swimming included the burying behaviour. This would appear to be a new description of the objective of burying behav-

our, where the male appeared to use the burying behaviour on top of a female (bury on) to elicit that the female swam from the bottom of the tank and the females appeared to use the burying behaviour (burying under) to reject a male. Previously the burying behaviour has only been associated with disturbances in the environment (to hide) and burying in at the end of a period of activity to begin a period of resting (Kruuk, 1963; Gibson, 2005). When the male’s active attention on top of the female achieved that the female began to swim from the bottom the male would immediately place itself under the female to begin the coupled swim. This positioning of the male under the female would be accepted and the couple would swim to the surface (Step 3) or be rejected and the female would swim away (failed spawn, type 1).

Failed spawning attempts have attracted little attention in other studies. In the present study, failed spawns (total 67) were 5.6 fold more frequent than successful spawns (12) over the 10 periods on which spawning was observed. No failed spawns were observed in periods without spawning. A similar ratio of failed to successful spawns was observed for winter flounder (Stoner *et al.*, 1999), which exhibited approximately 3 to 8 more failed spawns (avoidance behaviour) per hour compared to successful spawns. Other studies have simply acknowledged that instances were observed when the male’s approaches were rejected and the female swam away in common sole (Baynes *et al.*, 1994), eyed flounder (Konstatinou & Shen, 1995), wide-eyed flounder (Carvalho *et al.*, 2003) and greenback flounder (Pankhurst & Fitzgibbon, 2006). The observations that Senegalese sole spawn strictly in pairs and have a high ratio of failed spawns clearly indicated that mate selection was in progress between the males and females.

Senegalese sole did not appear to have any specific displaying behaviour in Step 2 as part of the mate selection process or courtship. The males of some flounder species, such as kobe flounder (Moyer *et al.*, 1985), largescale flounder (Manabe *et al.*, 2000), wide-eyed flounder (Carvalho *et al.*, 2003) and greenback flounder (Pankhurst & Fitzgibbon, 2006) take a position in front of the female and display, raising the head, arching the back and/or waving the pectoral fins before approaching to initiate the coupled swim. It is possible that the male Senegalese sole was displaying when moving over the back of the female. The close contact between the fish and the olfactory organs gave an opportunity for the exchange of conspecific pheromones for chemical communication. Carazo (2012) demonstrated that juvenile Senegalese sole in an electro-olfactogram (EOG) gave different olfactory responses to distinguish between mature and immature sole and

male and female sole, and Velez *et al.* (2007) demonstrated that the upper olfactory rosette gave a higher EOG response with conspecific-derived odorants compared to the lower olfactory rosette. This indicates that the female was well positioned in the courtship to recognise the male and receive a chemical display.

However, there was a clear opportunity for the males to display in the “following” behaviours that were observed in Step 1 before the “rest head” stage (Step 2) of the courtship. These “following” behaviours were only observed in periods with spawning and the fish participated in “following” behaviours before 10 out of the 12 spawns. Similar “following” behaviours were observed in the courtship of winter flounder (Stoner *et al.*, 1999). Both species displayed a behaviour where the fish swam in a procession, each following fish copying the swimming and turns of the lead fish. In the present study, the “following” behaviour at times resembled a chase or persecution, but had no clear aggression or competition associated and was most similar to a procession. The “following” behaviour appeared to be a display of swimming and the position of the fish in the procession, for example which fish was the lead fish and which fish followed. Altogether the three steps of the courtship indicated a passive process of mate selection between Senegalese sole males and females. The opportunities presented for mate selection were: a) strict paired spawning that gave the opportunity for the pair to select each other, b) swimming interactions using the entire area of the tank involving many males and some females that brought the fish into contact with each other, c) males rested their head and body on females to seek or confirm selection by females, d) a male resting the head of a female would shield her from the approaches of other males (guardian behaviour), e) males stimulated or encouraged the female to swim from the bottom, and f) females at times swam away from male advances and at times accepted the advances of a male to complete the courtship.

Paternity analysis of captive wild Senegalese sole demonstrated that 8.7% to 51.7% of breeders actually participated in spawning (Porta *et al.*, 2006b; Martin *et al.*, 2014) and that fidelity of spawning pairs appears to exist (Martin *et al.*, 2014). The courtship and mate selection description in the present study demonstrates how fidelity and restricted participation of breeders has been achieved. However, it is unclear why some breeders did not participate in spawning. Martin *et al.* (2014) described paternity in the same groups of fish used in the present behavioural study. In the years when the behavioural study was made, both tanks had similar percentage participation of females in the spawning with 21-25% of females participating in >80% of the

eggs by volume, 8-14% in the remaining <20% of eggs by volume and 42-43% not participating in spawning. Whilst male participation was 20-31% of males participating in >80% of the eggs by volume, 13-15% in the remaining <20% of eggs by volume and 23-47% not participating in spawning. The four *Bothidea* species (Moyer *et al.*, 1985; Konstantinou & Shen, 1995; Manabe *et al.*, 2000; Carvalho *et al.*, 2003) and the bastard halibut (Manabe & Shinomiya, 2001) were studied in the natural environment and the males were all observed to defend territories that included a harem of females or that females visited for courtship and spawning. This kind of behaviour would perhaps explain the differences observed in participation in spawning in Senegalese sole. Senegalese sole males were observed to defend access to females from other males and although the behaviour was more like a procession, the males were observed to chase each other. Senegalese sole juveniles have been observed to form hierarchies that explained differences in growth (Salas-Leiton *et al.*, 2010). It would appear possible that some kind of dominance of spawning was exerted by some Senegalese sole over the sole that did not spawn or had a low participation in spawning. Work is needed to examine the role on breeder participation in spawning of aspects such as: stocking densities, segregation of tanks into different areas and the segregation of spawning groups or pairs from non-spawning fish. Another approach would be to define or determine preference functions and choosiness to examine the relationship between mate selection and mating success (Edward, 2015). The present study indicates that preference functions, which would indicate which fish will be chosen as successful spawners, may be; participation or time participating in the “following” behaviours, position in the procession when making the “following” behaviour, total time that males rest the head on females. Such preference functions could be related to spawning success determined by parentage analysis (Edward, 2015), however, fish would need to be identified from the videos to make this kind of analysis.

The present description of successful spawning behaviour of captive wild Senegalese sole has relevance to the complete reproductive failure to produce viable eggs observed in hatchery reared Senegalese sole breeders. The description provides a base line of what successful spawning behaviour is for Senegalese sole. This baseline opens the possibility to study the reproductive behaviour of hatchery reared Senegalese sole breeders to identify differences and dysfunctions that could indicate why hatchery reared Senegalese sole breeders do not produce fertilised eggs. The description of successful spawning also highlights the complex process that particularly male breeders must execute

to achieve successful spawning and raises the question: Are hatchery reared breeders prepared or capable of completing these behaviours? Therefore, the description and ethogram provides the tools to study and quantify the reproductive behaviour of hatchery reared breeders to establish where or what aspects of the behaviour are missing to achieve the fertilisation of released ova.

In conclusion, the Senegalese sole exhibited a complex reproductive behaviour that was similar to other flatfish species. Spawning was strictly in pairs and the courtship description highlighted how males displayed, competed, and sort the attention of females. The females either accepted or rejected the male's advances. Opportunities existed for mate selection and for exclusion from the spawning process. Although these observations demonstrated the process by which breeders dominated spawning it remains unclear why certain breeders dominate spawning whilst other breeders do not participate. Lastly, the description of successful reproductive behaviour provides the opportunity to analysis the unsuccessful spawning behaviour of hatchery reared Senegalese sole breeders and identify what aspects of the reproductive behaviour are dysfunctional.

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