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Comparison of the reproductive cycles of two cryptic soles in the Adriatic Sea

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ABSTRACT

In the Adriatic Sea, two congeneric and cryptic soles coexist and share the same habitats. They are the common sole, *Solea solea*, one of the main Mediterranean fishery resources, and the Egyptian sole, *Solea aegyptiaca*. The present study investigated and compared their reproductive biology to obtain updated and original information useful for explaining their coexistence as distinct and reproductively isolated species and supporting stock assessment and management measures. The comparison of the reproductive cycle and strategy revealed similar and distinctive traits. Histological analysis indicated that both species had asynchronous ovaries and were batch-spawners. On the other hand, spawning activities occurred in late autumn or late winter-early spring in *S. solea* and *S. aegyptiaca*, respectively. Such a different reproductive timing may contribute to their sexual isolation and genetic distinction. Other pre-zygotic isolating processes have been hypothesized to act by preventing mating, such as the involvement of olfactory sense for intra-specific recognition. Fecundity estimates were similar between the two species (mean potential fecundity: 323,122 for *S. aegyptiaca* and 210,752 for *S. solea*, $P > 0.05$), but the oocytes produced by *S. aegyptiaca* were significantly smaller than those of *S. solea*. This study contributed to shedding light on the reproductive traits that may support the coexistence of these congeneric species and encouraged future investigations to understand their fitness in the Adriatic Sea.

1. Introduction

Investigations on reproductive biology, pertaining to the assessment of reproductive season, size at sexual maturity, fecundity, and reproductive strategy are fundamental to estimating the reproductive capacity of each fish and the spawning stock biomass (Murua et al., 2003). Studies on reproduction contribute to understanding the population dynamics and support stock assessment and management (Hunter et al., 1992). Even more so, the study of reproductive traits of sympatric cryptic or congeneric species is useful for defining their life histories, understanding their coexistence, and investigating hybridization processes (Ouanes et al., 2014; Compaire et al., 2018; Wu et al., 2021; Melo et al., 2023). Interspecific reproductive interactions can negatively affect a species' fitness,

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sometimes leading to its demographic displacement, but the evolution of distinct traits and mechanisms mediating reproductive interactions may allow species to coexist (Hochkirch et al., 2007; Gómez-Llano et al., 2021). In some cases, even small differences in sexual attributes, such as spawning timing or gametes development and characteristics, were observed to decrease the interspecific

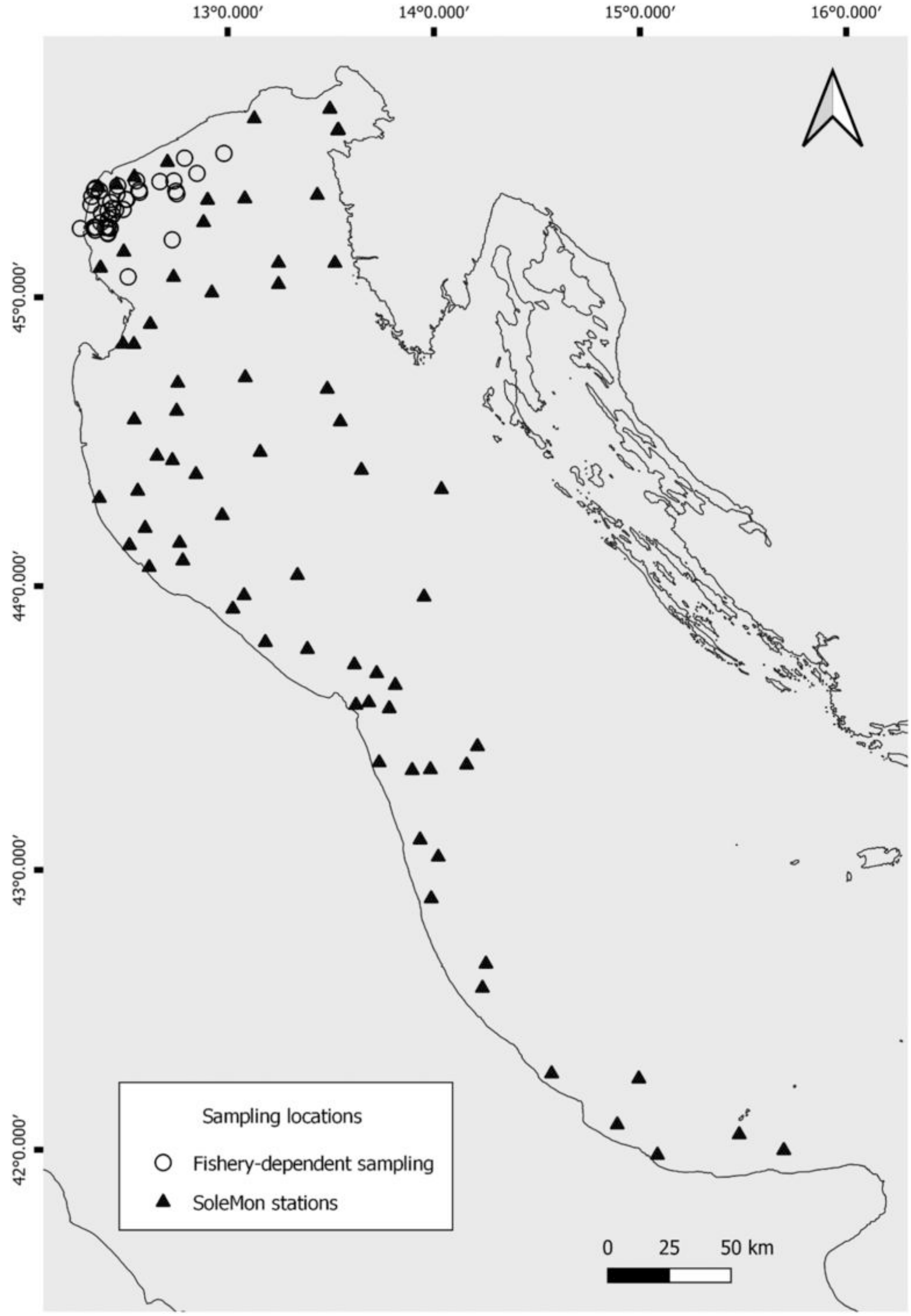

Fig. 1. Map of the study area (north and central Adriatic Sea) with fishery-dependent sampling locations (empty circles) and SoleMon survey stations (black triangles).

sexual interference between congeneric species or lineages of the same species (e.g., Wu et al., 2021; Melo et al., 2023). In the Adriatic Sea, the common sole, *Solea solea* (Linnaeus, 1758), one of the most valuable fishery resources, cohabits with a congeneric species, the Egyptian sole, *Solea aegyptiaca* (Chabanaud, 1927). As elsewhere in the Mediterranean (Boukouvala et al., 2012), fishermen misidentify them because of their similar external morphology and land both species as *S. solea*. Therefore, no official data are available for the *S. aegyptiaca* population of the Adriatic Sea.

The *S. solea* is annually monitored in the northern-central Adriatic Sea to assess the stock status for fishery management purposes (Masnadi et al., 2020; FAO-GFCM, 2022; STECF, 2022; Carbonara et al., 2023). The last estimates stated that the species biomass is increasing and fishing mortality (F) is below F at the Maximum Sustainable Yield (F_{MSY}) (STECF, 2022). The species is considered resilient enough due to the higher exploitation of juveniles than adults because of offshore refuges. The presence of a peculiar benthic community in the offshore areas dominated by holothurians and bryozoans protects large and old spawners from trawl fishing activities (Santelli et al., 2017). For this reason, these areas have been defined to be “sole sanctuaries” (Scarcella et al., 2014). The biology and ecology of *S. aegyptiaca* are primarily investigated in the south and eastern waters of the Mediterranean Sea (Mehanna, 2007; Ahmed et al., 2010; Gabr, 2015; Desouky, 2016; Khalifa et al., 2019), where it is more frequent and abundant, and is considered an important fishery resource (Mehanna, 2007; Gabr, 2015). In general, the two species are considered reproductively and genetically isolated everywhere they were observed and investigated in sympatry (Quignard et al., 1984; She et al., 1987; Borsa and Quignard, 2001). Conversely, the *S. aegyptiaca* is more closely related to the Senegalese sole, *Solea senegalensis* (Kaup, 1858), and hybrid specimens were observed and studied in Tunisian waters (Goucha et al., 1987; She et al., 1987; Ouanes, 2011).

In the Adriatic Sea, reproductive biology has been investigated only for *S. solea*, and the studies date back to the ‘80 s and 2000s (Piccinetti and Giovanardi, 1984; Vallisneri et al., 2000 and 2001). To fill these gaps, this study focused on the reproductive strategies of *S. aegyptiaca* and *S. solea* in the Adriatic Sea. The main aim was to obtain updated and original information useful for explaining their coexistence as distinct and reproductively isolated species and supporting assessment and management actions on fishery-valuable species. In more detail, the study attempted to 1) validate the macroscopic classification of maturity stages through histological analysis, 2) describe the gonad cycle, 3) define the timing and duration of the reproductive season and 4) estimate fecundity and length at sexual maturity.

2. Materials and methods

2.1. Study area and sampling activities

Fishery-dependent and independent samplings were conducted in the Adriatic Sea across 2021. Due to bureaucratic fulfillment, the sample activities started with a month delay. *S. solea* and *S. aegyptiaca* were collected monthly from February to December 2021 from commercial landings of different fishing gears (otter-trawl, *rapido* trawl, and gillnet) at the Chioggia fishery market (northwestern Adriatic). The sampling area was chosen based on evidence of the persistence of *S. aegyptiaca* coming from SoleMon (Solea Monitoring Project) surveys and personal field observations. Fishermen provided geographic information on fishing spots. Further specimens were sampled during the SoleMon *rapido* trawl survey 2021. This survey is carried out annually in the fall according to a depth-stratified sampling scheme with random allocation of stations over the central and northern Adriatic Sea (Grati et al., 2013; Anonymous, 2019). The study area and sampling stations are shown in Fig. 1.

2.2. Sample processing

The external characteristics of the caudal peduncle and the color pattern of the right side were evaluated to identify the two species, as described by Sabatini et al. (2018). All ambiguous specimens were excluded from the analyses.

Overall, 30–60 individuals per species, representing the entire size range, were selected monthly. Due to the low abundance of *S. aegyptiaca* and the change of target species and fishing grounds by commercial fishing over the months, relatively few individuals were available in the spring months. For each specimen, total length (L, to the nearest millimeter below), wet weight (W_W, 0.1 g), sex (female, male, undetermined), and gonad maturity stage were recorded. Gonad maturity stage was determined macroscopically following Holden and Raitt (1974) combined with ICES (ICES, 2010; 2012) maturity scales composed of 5 stages: 1) immature, 2) maturing virgin/regenerating, 3) ripening, 4) spawning, 5) spent. The male classification was more complicated due to slight gonad changes during development (Fig. S1).

Female and male gonad subsamples, representative of the whole size range at each stage, were selected and stored in Dietrich solution (900 ml distilled water; 450 ml 95 % ethanol, 150 ml 40 % formaldehyde, and 30 ml acetic acid) at least 60 days before the following histological analysis. Histological preparation followed the standard protocol of dehydration, clearing, embedding, sectioning (7 μm), and staining (Mayer’s hematoxylin-eosin; Pearse, 1985). The histological sections were observed under a microscope (Leica DM4000B) at different magnifications. The gonadal development was assigned to the following phases, according to the criteria of Brown-Peterson et al. (2011): immature, developing (with an early-developing subphase), spawning capable (with an actively spawning subphase), regressing, and regenerating.

Ripening or spawning females (3 and 4 stages) were selected from the whole size range during the spawning season for fecundity estimates. From each of these specimens, right and left gonads were weighed and stored separately for histological analysis and fecundity estimation. The gravimetric method (Murua et al., 2003) was applied to count and measure advanced (yolked) oocytes from a gonad portion corresponding to 1–2 % of gonad weight. The number and size frequency of oocytes usually do not differ between the two gonads (Hunter et al., 1985). As a consequence, the right ovary was arbitrarily chosen for fecundity estimate. The gonad subsample was placed in a Petri dish with a dark background and the oocytes were manually separated from the ovarian lamellae under a stereomicroscope (Leica M205C) with reflected light (10x magnification). A series of oocyte images were taken using a charge-coupled device camera (Leica DFC420) linked to a digitized computer video system (Leica Application Suite 4.3.0) and analyzed to count and measure the diameter of all oocytes using the application tools.

2.3. Data analysis

2.3.1. Histological and macroscopic gonadal staging

Histological analyses were carried out on 115 samples (47 *S. aegyptiaca* and 68 *S. solea*) to assess gonadal development and to validate the macroscopic stage assignments (i.e. consistency between macroscopic and microscopic maturity scales, Table S1). The monthly percentage frequency of the macroscopic reproductive stages was calculated only for females of both species, due to the difficulties of the male gonad staging. The analysis was carried out using the R studio *ggplot2* package.

2.3.2. Gonadosomatic index, condition factor, and hepatosomatic index

To evaluate the reproductive cycle during the year, the gonadosomatic index was calculated for both sexes each month according to the following equation: $GSI = W_g / W_W * 100$ (W_g = gonads weight, W_W = total body weight). Immature specimens were excluded from the analyses.

The relative condition factor: $K_{rel} = W_W / aL^b$ (Le Cren, 1951) was calculated for each sex of both species to investigate the relative condition of individuals (a and b were estimated from sample length-weight relationships, Sabatini et al., 2025), compensating for changes in form or condition during the ontogeny (Blackwell et al., 2000; Froese, 2006), throughout the sampling period. Since trends were similar, the condition factor was calculated using total weight instead of eviscerated weight.

The hepatosomatic index: $HSI = W_{liver} / W_W * 100$ was determined as a proxy of metabolic activity (Papiol et al., 2014). The Kruskal-Wallis test was applied to investigate sex-related and seasonal variability of the three indices. When the test was significant (significance level set at $P < 0.05$), a post hoc analysis (the Dunn test) was performed to determine which months differed (Bonferroni's correction was used to adjust the p-values for multiple comparisons). All these analyses and graphs were carried out using the R studio *stats*, *FSA*, and *ggplot2* packages.

2.3.3. Size at sexual maturity

Fish size at sexual maturity (L_{50}, representing the size at which 50 % of the individuals reach sexual maturity) was calculated only for females of *S. solea*, due to the difficulties in the macroscopic staging of males and the scarce number of small specimens of *S. aegyptiaca*. The estimate was based on the identification of mature (soles that certainly will or have already spawned, i.e. those at 3–5 stages) and immature individuals collected during the reproductive period (comprised between the appearance of developing and post-spawning individuals). The logistic curve was fitted to the fish size and maturity stage data pairs using the software Past 4.02 (Hammer et al., 2001). Only the minimum size at sexual maturity was reported for males of *S. solea* and both sexes of *S. aegyptiaca*.

2.3.4. Fecundity

For fecundity estimation, a threshold size of 200 μm was selected as the minimum diameter of vitellogenic oocytes based on oocyte measurements carried out on histological sections. Having both species a determinate fecundity (Horwood and Greer Walker, 1990; Abdel-Aziz, 1994; Murua and Saborido-Rey, 2003), the potential annual fecundity (the total number of vitellogenic oocytes matured per female per year, uncorrected for atretic losses) and the batch fecundity (the number of hydrated oocytes released in each spawning event) were estimated. Samples without postovulatory follicles (POF) were considered suitable for potential fecundity calculation. The number of vitellogenic or hydrated oocytes counted in the subsample was multiplied by the ratio between the gonad total weight and the subsample weight. The relative fecundity (potential or batch fecundity divided by female gutted weight) was calculated (Hunter et al., 1992). The relationship between fecundity and individual size was evaluated by fitting the linear regression to $\log_{10}$-transformed data. The assumptions of normality and homogeneity of variances were previously tested using Shapiro-Wilk and Levene's tests, respectively. Analysis of covariance (ANCOVA) was applied to investigate the differences in fecundity between the two species considering the effect of body length. The oocyte diameters were compared between the two species using the Mann-Whitney U test. The significance level was set at $P < 0.05$. All these analyses were carried out using the R studio *stats* and *car* packages.

2.4. Ethical statement

Animal manipulation complied with the guidelines of the European Union Directive (2010/63/EU) and the Italian Legislative Decree 26 of March 4, 2014 "Attuazione della Direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici". Ethical review and approval were not required because the specimens analyzed came from landings of fishery and survey activities, so no live animals were subject to any experimental manipulation, in line with the Explanatory Note of the Italian Ministry of Health's Directorate-General for Animal Health and Veterinary Medicinal Products (DGSAF) of July 26, 2017.

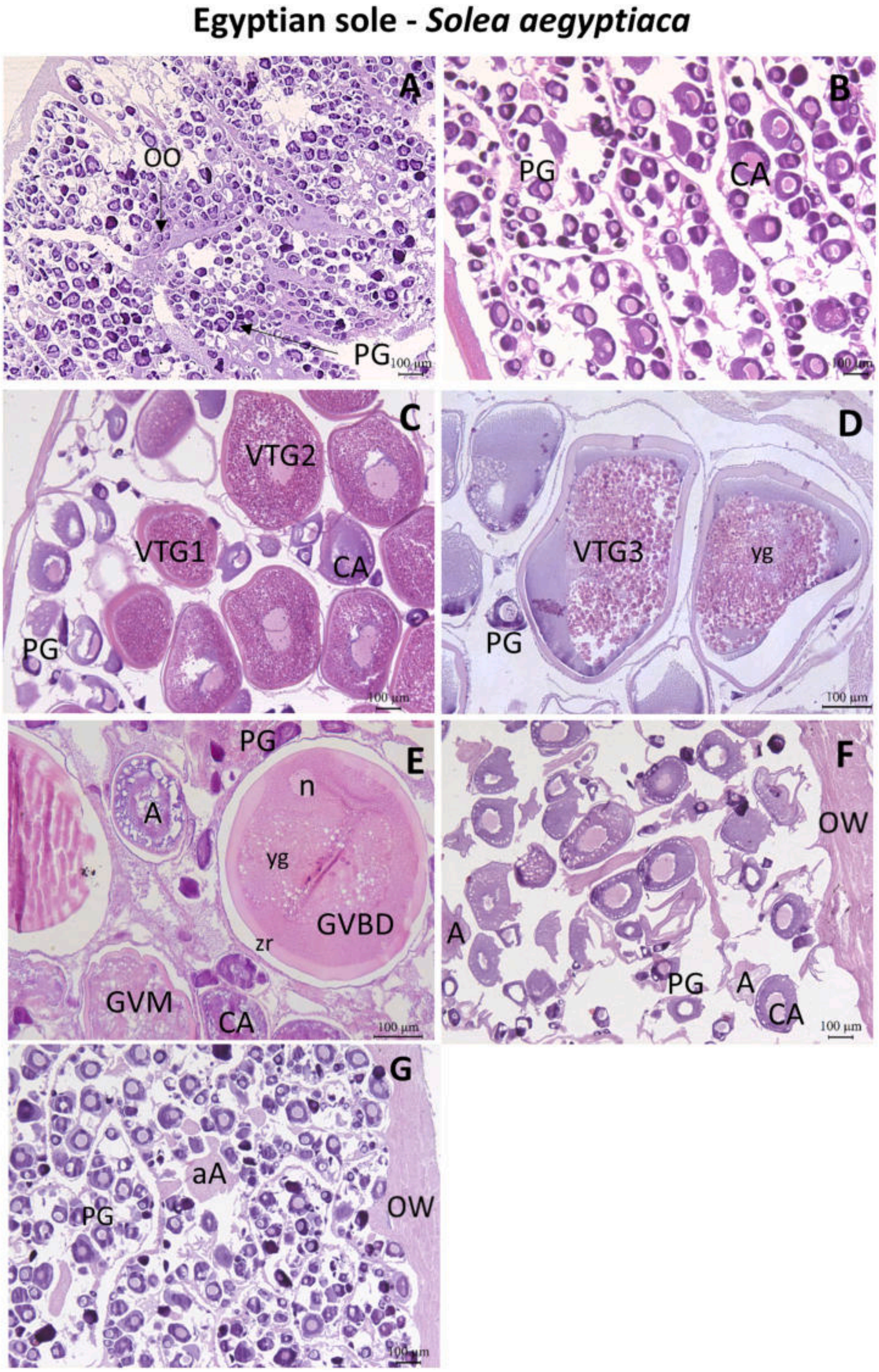

Fig. 2. Photomicrographs of ovarian histology illustrating the reproductive phases of Egyptian sole, *Solea aegyptiaca*. A: immature; B: early-developing; C: developing; D: spawning capable; E: actively spawning; F: regressing; G: regenerating. OO = oogonia; PG = primary growth oocytes; CA = cortical alveolar oocytes; VTG1 = primary vitellogenic oocytes; VTG2 = secondary vitellogenic oocytes; VTG3 = tertiary vitellogenic oocytes; GVM = germinal vesicle migration; GVBD = germinal vesicle breakdown; POF = post-ovulatory follicles; OW = ovarian wall; aA = advanced atresia; A = atresia; n = nucleus; yg = yolk granules; yc= yolk coalescence; zr = zona radiata. Hematoxylin and eosin staining.

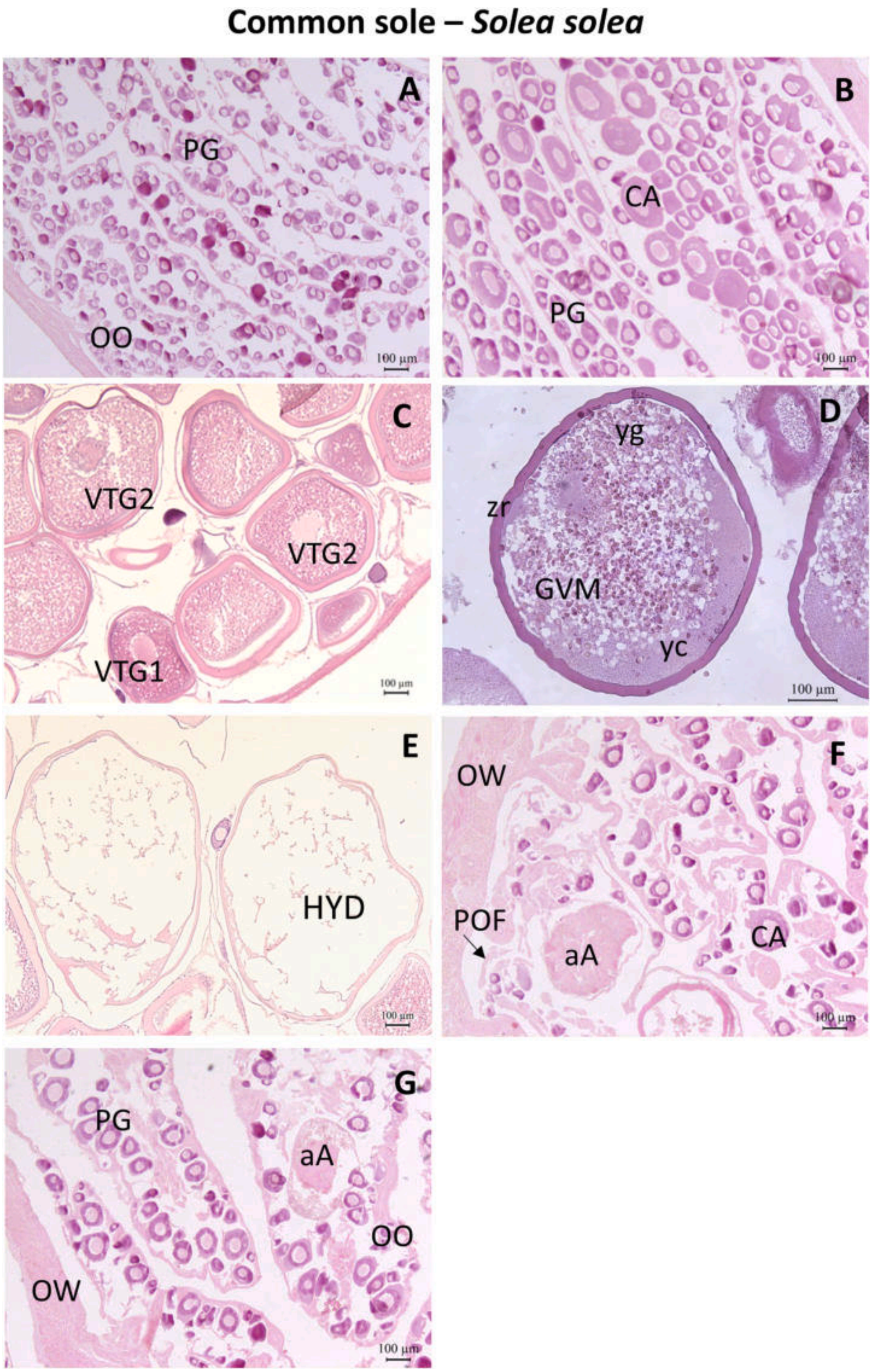

Fig. 3. Photomicrographs of ovarian histology illustrating the reproductive phases of common sole, *Solea solea*. A: immature; B: early-developing; C: developing; D: spawning capable; E: actively spawning; F: regressing; G: regenerating. OO = oogonia; PG = primary growth oocytes; CA = cortical alveolar oocytes; VTG1 = primary vitellogenic oocytes; VTG2 = secondary vitellogenic oocytes; VTG3 = terziary vitellogenic oocytes; GVM = germinal vesicle migration; GVBD = germinal vesicle breakdown; HYD = hydration; POF = post-ovulatory follicles; OW = ovarian wall; aA = advanced atresia; A = atresia; n = nucleus; yg = yolk granules; yc= yolk coalescence; zr = zona radiata. Hematoxylin and eosin staining.

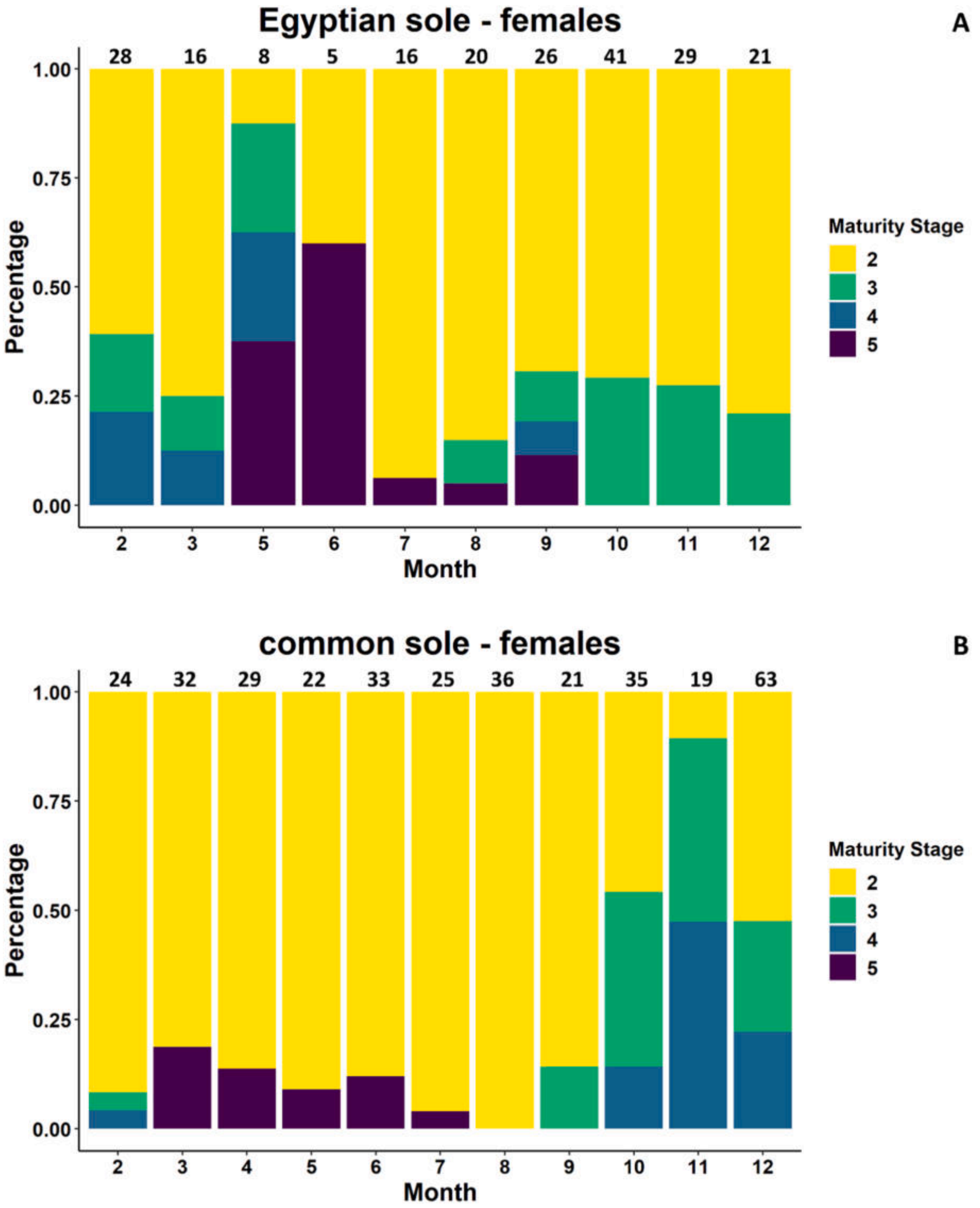

Fig. 4. Monthly percentage frequency of female macroscopic reproductive stages (from stage 2–5) of *Solea aegyptiaca* (A) and *Solea solea* (B). Numbers on the top of columns indicate the abundance of specimens per month.

3. Results

3.1. Histological and macroscopic gonadal staging

Histological analysis was conducted on 47 *S. aegyptiaca* (28 females and 19 males) and 68 *S. solea* (42 females and 26 males). Macroscopic and histological maturity classifications corresponded in 70 % of the analyzed samples, 33 out of 47 *S. aegyptiaca*, and 48 out of 68 *S. solea*. Mismatched estimates were more frequent for males (9 cases out of 14 in *S. aegyptiaca* and 13 out of 20 in *S. solea*). In this case, the macroscopic classification underestimated the spawning (macroscopic stage 4) and spent (macroscopic stage 5) gonads. In females, the mismatch was observed outside the spawning period when some gonads were externally considered spent, but histology recognized them as regenerating; and during the pre-spawning and spawning period, when gonads were macroscopically classified as ripening (macroscopic stage 3) but they were identified as spawning capable or regressing by histology and vice versa.

The histological analysis described five gonadal development phases and two subphases for females of both species (Figs. 2 and 3 and Table S2). In the samples analyzed, atretic oocytes (A) were observed starting from the developing phase, and advanced atresia (aA) was present in regressing and regenerating ovaries.

Gonads in the ripening phase (macroscopic stage 3) were observed in both species at the end of summer (Fig. 4A and B). Spawning

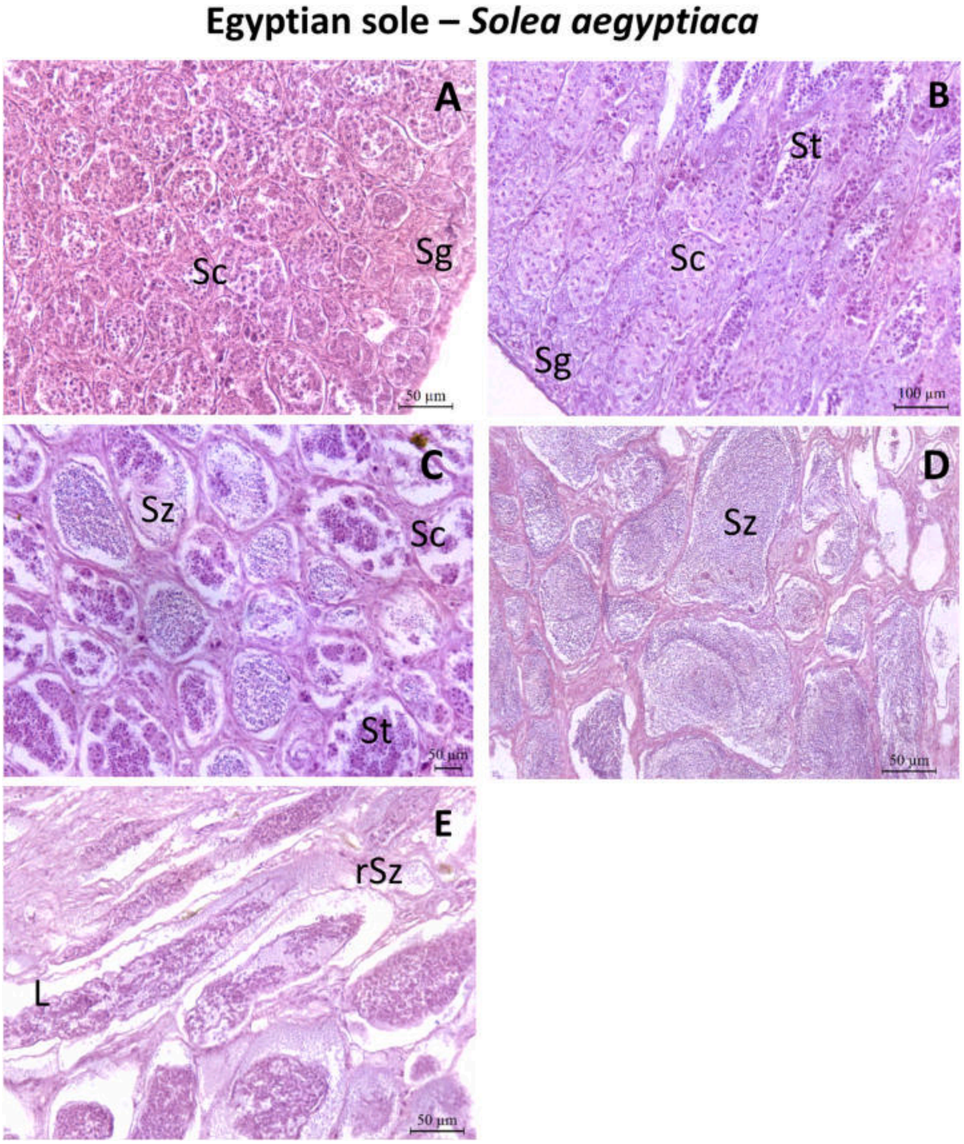

Fig. 5. Photomicrographs of testis histology illustrating the reproductive phases of Egyptian sole, *Solea aegyptiaca*. A: early-developing; B: developing; C-D: spawning capable; E: regressing. Sg = spermatogonia, Sc = spermatocyte, St = spermatid, Sz = spermatozoa, rSz = residual spematozoa, L = lumen of lobule. Hematoxylin and eosin staining.

gonads (macroscopic stage 4) appeared in specimens of *S. solea* collected from October to February, and *S. aegyptiaca* sampled between February and May. The presence of Egyptian soles at stage 4 in September was due to only two specimens, considered outliers in the following GSI analysis, which anticipated the development (Fig. 7A). Generally, the fewer Egyptian soles caught influenced the less clear stage distribution. Females moved into the spent stage (macroscopic stage 5) in March for *S. solea* and in May for *S. aegyptiaca*. Finally, the gonads reached the regenerating phase (macroscopic stage 2) during spring and summer.

Only three development phases and one subphase were observed for males (Figs. 5 and 6 and Table S3). Those macroscopically classified immature (stage 1) selected for histological analyses showed to be early-developing or developing, so the immature phase was not present in the samples. It seemed that males developed earlier in time than females, the developing phase started in the summer. Generally, the actively spawning subphase is only macroscopically distinguishable in males, when milt is released with gentle pressure on the abdomen, but this is not the case for soles. Due to the tiny dimension, only by cutting the gonad the tissue fluid consistency may be observed (ICES, 2012). The regenerating phase was not described here because no regenerating male was found among the samples selected for histological analysis due to the difficulties in macroscopic stage recognition.

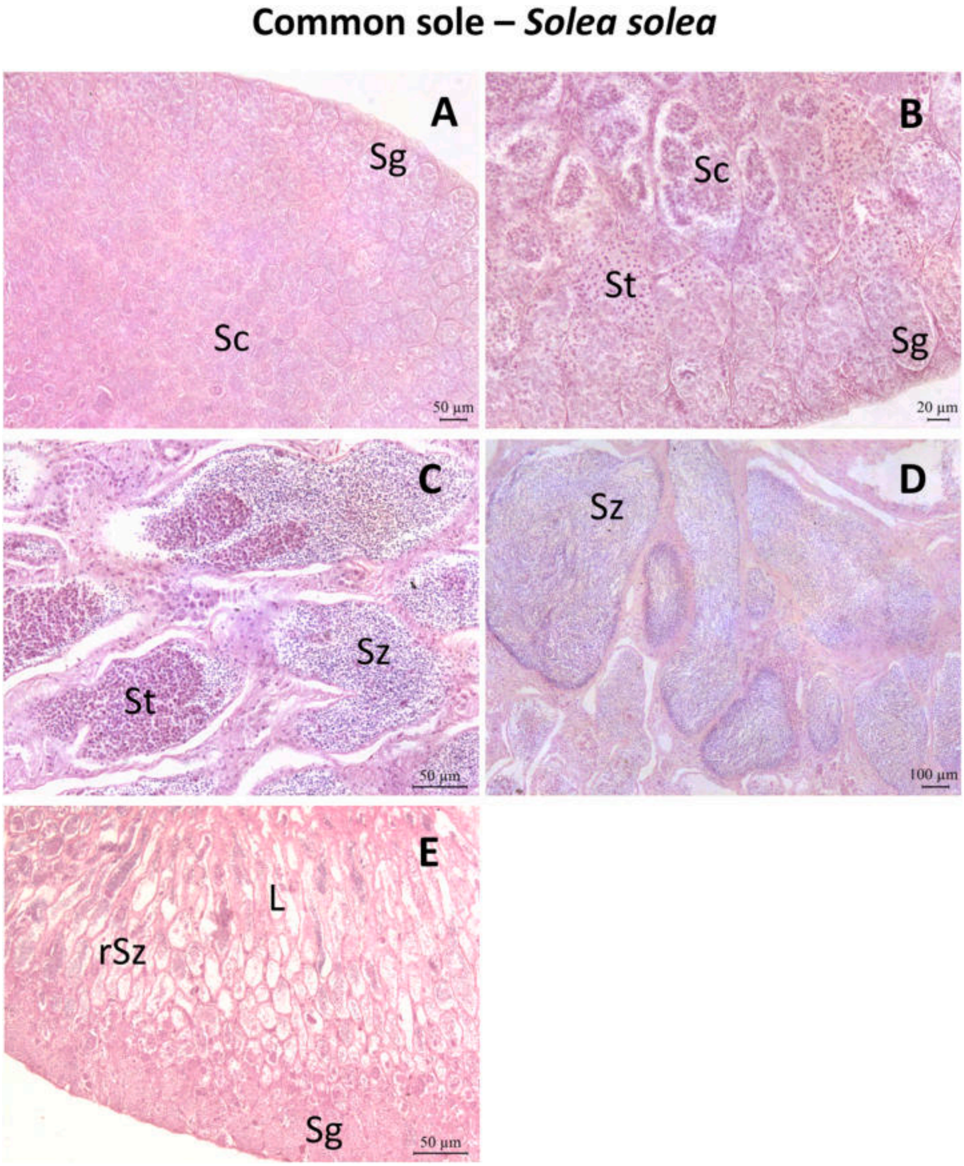

Fig. 6. Photomicrographs of testis histology illustrating the reproductive phases of common sole, *Solea solea*. A: early-developing; B: developing; C-D: spawning capable; E: regressing. Sg = spermatogonia, Sc = spermatocyte, St = spermatid, Sz = spermatozoa, rSz = residual spematozoa, L = lumen of lobule. Hematoxylin and eosin staining.

3.2. Gonadosomatic index

The gonadosomatic index monthly trend of females of *S. solea* indicated that the most intense spawning activity took place between October and December, peaking in November (average GSI 10 %, maximum 18 %) (Fig. 7A). The Kruskal-Wallis test confirmed significant differences between this period and the rest of the year (K-S chi-squared = 93.68 d.f. = 10 $P < 0.0001$). No data were available for January, but it could be assumed that there were still active individuals. Regarding GSI, males showed a lower reproductive effort than females (Fig. 7B). Also in this case, the values of the last three months of the year, particularly November, were significantly higher than the others (K-S chi-squared = 92.17 d.f.= 10 $P < 0.0001$). The macroscopic classification was complicated due to the reduced changes during development (Fig. S1). GSI values ranged between 0.002 % and 0.6 %, and the temporal trend was similar to that of females, with an increase in late autumn and a reduction at the end of winter. Thus, the reproductive period was spread over a relatively long period between October and March.

The monthly trend of *S. aegyptiaca* GSI was less clear due to fewer or no samples in some months. Despite this, it showed a shift of

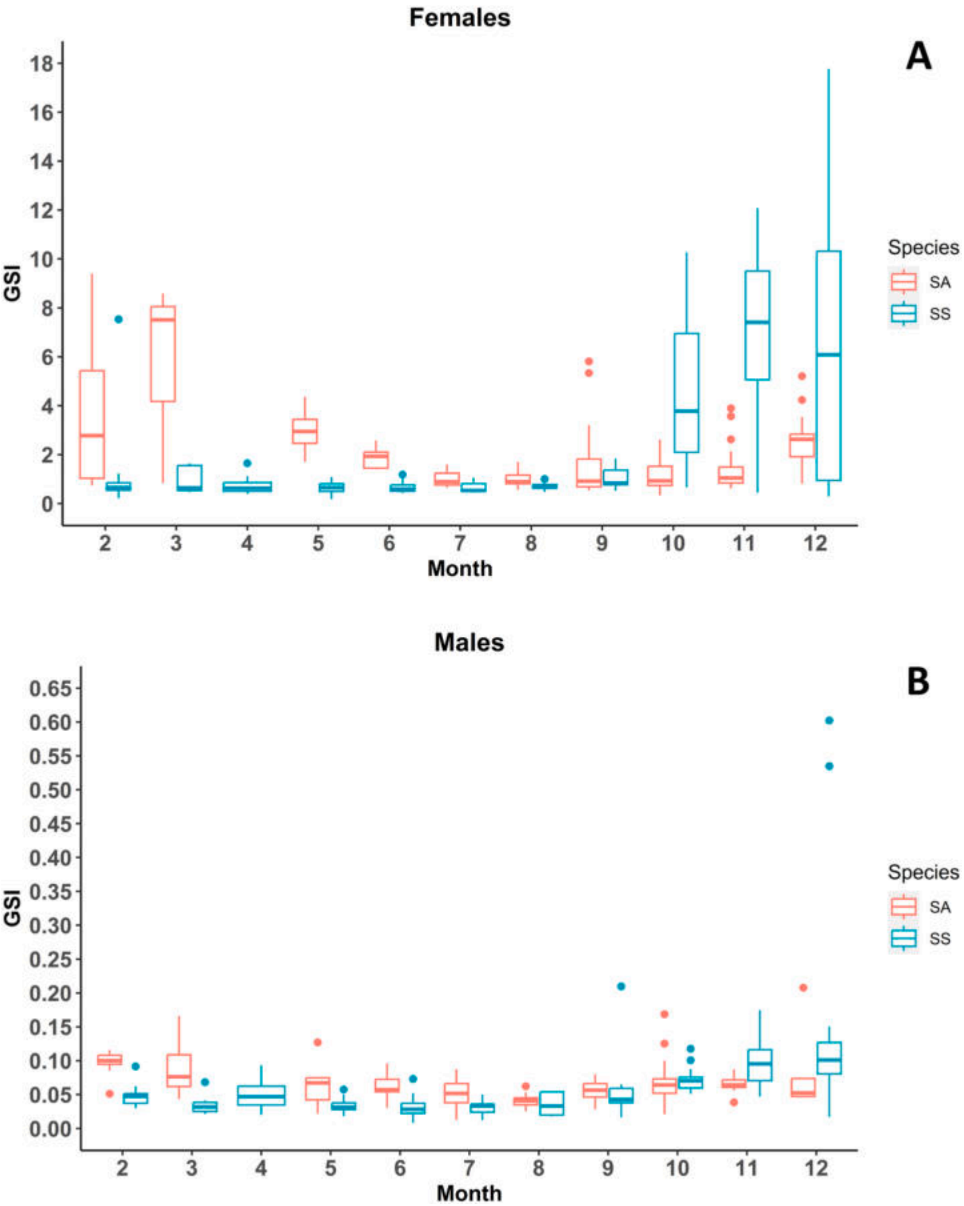

Fig. 7. Monthly trend (February is represented by 2 and December 12) of the gonadosomatic index (GSI) of females (A) and males (B) of Egyptian sole, *Solea aegyptiaca* (SA, red), and common sole, *Solea solea* (SS, light blue). The box represents the interquartile range (between the first and the third quartiles), the horizontal line is the second quartile, the whiskers indicate max(x[x < Q3 + 1.5 * IQR]) and min(x[x > Q1 - 1.5 * IQR]), and the points are the outliers.

the spawning activity (mean GSI 7 %, maximum 9 %) towards late winter and spring compared to the *S. solea* (Fig. 7A). Males had a similar trend to females with lower GSI index values (0.01 – 0.2 %) (Fig. 7B). The Kruskal-Wallis test highlighted significant differences between months in both sexes (females: K-S chi-squared = 54.57 d.f. = 9 $P < 0.0001$, males: K-S chi-squared = 60.38 d.f. = 9 $P < 0.0001$). The post hoc comparisons showed a less clear picture, probably due to the small number of samples in several months. In females, the index was significantly higher in February, May, and December than in other months. In males, October, November, February, and March values were significantly higher than summer months. It is possible to hypothesize that the reproductive season started in January, the peak of spawning was reached at the end of winter and early spring (March-April), and ended in June.

3.3. Relative condition factor

The best body conditions were reached at the end of summer and autumn for both sexes of *S. aegyptiaca* (Fig. S2A), and at the end of spring and fall for females of *S. solea* (Fig. S2B). The monthly trend was less clear for males of *S. solea*. The Kruskal-Wallis test showed significant temporal differences in both species (SA females: K-S chi-squared = 60.99 d.f. = 10 $P < 0.0001$, SA males: K-S chi-squared = 56.42 d.f. = 10 $P < 0.0001$, SS females: K-S chi-squared = 68.51 d.f. = 10 $P < 0.0001$, SS males: K-S chi-squared = 23.06 d.f. = 10 $P = 0.011$). The post hoc tests indicated significant differences mainly between the last three months of the year and the others, particularly in females. In *S. solea* males, no significant comparisons were observed.

3.4. Hepatosomatic index

The hepatosomatic index showed a quite similar monthly trend to the condition factor (Fig. S3A and B). In females, the higher values at the end of spring and in autumn were significantly different than the lower values reached at the end of winter and during summer (Kruskal-Wallis chi-squared: SA females = 39.64 d.f. = 9 $P < 0.0001$, SS females = 61.77 d.f. = 10 $P < 0.0001$). The index variations in males were significantly higher only in August and in May for *S. aegyptiaca* and *S. solea*, respectively (Kruskal-Wallis chi-squared: *S. aegyptiaca* = 40.28 d.f. = 10 $P < 0.0001$, *S. solea* = 49.95 d.f. = 10 $P < 0.0001$).

3.5. Size at sexual maturity

The size at sexual maturity L_{50} was estimated only for females of *S. solea* from specimens collected between September and March. The value corresponded to 25 cm, size reached between the first and the second year of life (Fig. S4). The parameter was not evaluated for males because of the difficulties in the macroscopic staging (described in paragraph 3.1). The smallest females with developing gonads (macroscopic stage 3) were observed at 23 cm, whereas males at 18 cm. The smallest *S. aegyptiaca* females and males with developing gonads were 20 cm and 18 cm, respectively.

3.6. Fecundity

Fecundity was estimated for 12 *S. aegyptiaca* (*L* 27.2 – 35.4 cm, mean 31.7 cm) and 11 *S. solea* (*L* 23.7 – 37.1 cm, mean 27.4 cm). Values were similar between species (Table 1). *S. aegyptiaca* batch fecundity and relative batch fecundity values referred to the single individual found with hydrated oocytes. The other females classified as spawning capable were found to be at the beginning of the hydration process with a quite low number of hydrated oocytes. The total number of vitellogenic oocytes was positively related to body size. The equation was $F = 0.045L^{4.55}$ ($R^2 = 0.77$ $P < 0.001$) for *S. aegyptiaca* and $F = 0.094L^{4.31}$ ($R^2 = 0.83$ $P < 0.001$) for *S. solea*. The comparison of fecundity between the two species did not show significant differences (ANCOVA $F_{1,20} = 0.118$ $P = 0.738$).

The oocyte size frequency distributions showed two or three modes representing the different developing stages. The first mode corresponded to oocytes from the previtellogenesis to the early vitellogenesis phase, the second to the advanced vitellogenesis stage, and the third to the hydrated oocytes (Fig. 8, S5, and S6 and Table S4). The size frequency distributions of females in earlier development stages showed more overlap between oocyte groups than those in the advanced phase. The size comparisons of oocytes belonging to each mode showed significant differences between the two species. The previtellogenic - early vitellogenic oocytes ($Z_{21} =$

Table 1
Potential fecundity (F), batch fecundity (F_b), and relative fecundity (Relative F and Relative F_b) estimates for 12 Egyptian sole, *S. aegyptiaca*, and 11 common sole, *S. solea*. Mean (± standard deviation), minimum, and maximum values were reported.

	Egyptian sole			**common sole**		
	mean (S.D.)	**min**	**max**	**mean (S.D.)**	**min**	**max**
Potential Fecundity (F)	323,122 (± 139,405)	164,151	484,095	210,752 (± 181,158)	79,854	693,371
Batch Fecundity (F_b)	10,969 -	-	-	12,382 (± 4889)	3886	16,350
Relative F	886 (± 145)	756	1122	894 (± 240)	592	1229
Relative F_b	44 -	-	-	55 (± 23)	26	78

-2.58 $P = 0.009$), advanced vitellogenic oocytes ($Z_{21} = -3.39$ $P = 0.0003$), and hydrated oocytes ($Z_{11} = -3.26$ $P = 0.0014$) were significantly larger in *S. solea* than in *S. aegyptiaca* (Fig. 8). The maximum diameter of hydrated oocytes was 1280 µm and 1770 µm in *S. aegyptiaca* and *S. solea*, respectively.

4. Discussion

This study investigated the main reproductive traits, such as gonadal development, reproductive season, and fecundity to provide updated information on the reproductive biology of two sympatric congeneric soles. Several characteristics overlapped, but the spawning timing and the oocyte dimensions differed, suggesting fundamental divergent traits that can promote reproductive isolation between the two species.

The assigned macroscopic maturity scale corresponded to the real developing stage in 70 % of the analyzed samples. Differences in gonad stage classification between macroscopic and histological analyses were more frequent for males (22 out of 30 mismatched classifications), primarily due to the overestimation of the immature stage and the underestimation of the spawning and spent gonads. Indeed, the very small kidney-shaped gonads undergo faint changes in volume and shape during maturation (Fig. S1). It is tough to distinguish even between a ripening or spawning gonad because milt does not run freely and only cutting the gonad may show a different consistency (more fluid in the latter phase) (Bromley, 2003; ICES, 2012), which is time-consuming during field activities. A problem in the macroscopic staging of females was observed during the months after the spawning activity, with gonads in the regenerating phase often classified as spent. It is reported that partially mature, post-spawning, or resting gonads are usually confused (Murua et al., 2003) and that, to obtain more reliable estimates, the staging should be done from two months before until the end of the spawning season (ICES, 2012). Some mismatched cases occurred during pre- and spawning activity, when gonads moved from the ripening to the spawning phase or from spawning to post-spawning. Since the presence/absence of tertiary oocytes is the histological criterion for distinguishing between developing and spawning capable, it may be possible to misclassify some gonads at these stages macroscopically. In some cases, the external observation of hydrated oocytes could fail (Horwood and Greer Walker, 1990). Furthermore, as both species are batch spawners, spawning gonads at the advanced phase may be confused with the transition to the post-spawning phase, gonads that could be considered partially spent (Brown-Peterson et al., 2011). The applied macroscopic staging is a valid tool to describe female gonad development of soles, whereas male classification is often inaccurate due to the gonad morphology so it should be used more carefully.

Through the histological analysis, it was possible to follow the gametogenesis of both sexes and infer the reproductive strategies of *S. solea* and *S. aegyptiaca* in the Adriatic. Seven gonadal development phases were described for females and four for males. Female gametogenesis indicated that both species had asynchronous ovaries, with different oocyte development phases simultaneously, and were batch-spawners, releasing eggs in batches over a protracted spawning period, as confirmed by the GSI monthly trend. Testes were the unrestricted spermatogonial lobular type, with spermatogonia distributed along the entire lobules (Uribe et al., 2015). Males seemed to start the gonad development before females, as they were always ready to spawn during the spawning season and wait for females to mature (ICES, 2010).

In the present study, the spawning activity of *S. solea* was observed in late autumn, between October and December, when the bottom temperature ranged between 9 and 11 °C. A shorter and earlier period than what other authors reported for the Adriatic Sea, namely December-March (Vallisneri et al., 2000 and 2001) and November-March (Piccinetti and Giovanardi, 1984), and the western

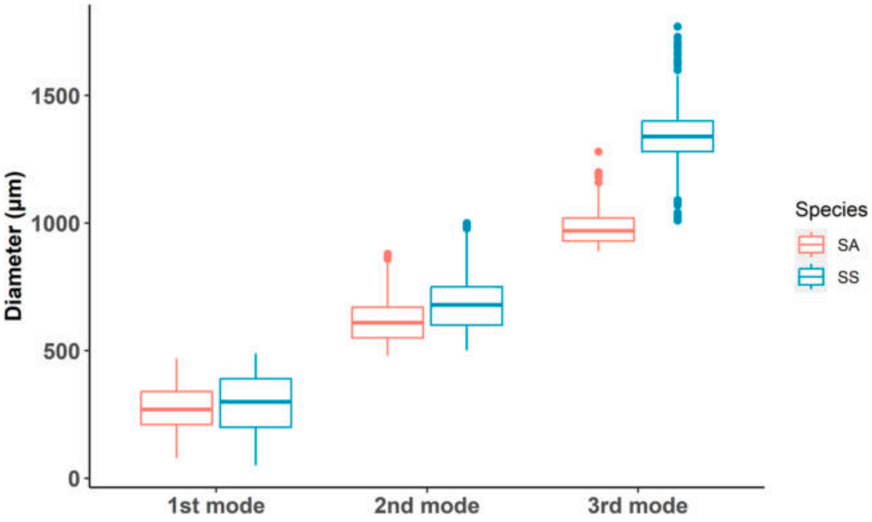

Fig. 8. Boxplots representing the diameter (µm) of each oocyte mode compared between Egyptian sole, *Solea aegyptiaca* (SA, red), and common sole, *Solea solea* (SS, light blue). The box represents the interquartile range (between the first and the third quartiles), the horizontal line is the second quartile, the whiskers indicate max(x[x < Q3 + 1.5 * IQR]) and min(x[x > Q1 - 1.5 * IQR]), and the points are the outliers.

Mediterranean, December-April in Spain (Ramos, 1982) and November-March in the Gulf of Lion (Shehata, 1984). In the eastern Mediterranean, the spawning period was shifted to early spring, April-May in Turkey (Türkmen, 2003) and April-June in the Marmara Sea (Slastenenko, 1956 in Tsikliras, 2010), except for Egypt, where the period extended from November to April (Mehanna, 2014). The reproductive season of *S. aegyptiaca* in the Adriatic basin seemed to append between the end of winter and early spring when the bottom temperature ranged between 12 and 15 °C. It partially corresponded to what was reported in the southeastern Mediterranean, where the species was classified as a winter spawner. In Egypt, the species was observed active between January and June, with a peak in January (Ahmed et al., 2010) or December and February (Abdel-Aziz, 1994), and in Tunisia, the reproduction extended from October to February, with a peak in November-December (Khalifa et al., 2019).

The two species have always been considered sexually isolated, wherever they live in sympatry (Quignard et al., 1984; She et al., 1987; Borsa and Quignard, 2001), differently from *S. aegyptiaca* and Senegalese sole (*Solea senegalensis*), for which a hybridization area has been identified in the northern Tunisian waters (Goucha et al., 1987; She et al., 1987; Ouanes, 2011). The difference in the reproductive periods observed in this study, particularly during the spawning peak months, may contribute to sexual isolation and genetic distinction (Sabatini et al., 2018) of the two species in the Adriatic Sea. Temporal segregation of spawning can promote the coexistence of related species reducing sexual interference. Previous studies reported temporal partitioning during the reproduction of congeneric or lineages of the same species living in sympatry. All the authors hypothesized the role of temporal segregation in species co-occurrence (Compaire et al., 2018; Wu et al., 2021; Melo et al., 2023). Furthermore, other pre-zygotic isolating processes could act by preventing mating. Reproductive isolation can occur through habitat specialization (e.g., the spatial segregation of the species during reproduction), species recognition mechanisms, which can involve behavioral, auditory, or chemical signals, and fertilization, when gametes are not receptive between the species (Bickford et al., 2007; Knowlton, 1993; Palumbi, 1994). The reproductive areas of *S. solea* correspond to the entire spatial distribution of adults with some concentration zones (Piccinetti and Giovanardi, 1984), such as the “sanctuary area” in the central-eastern part of the basin (Scarcella et al., 2014). Therefore, spatial segregation between the two species is unlikely and other segregation processes, e.g., sensorial system, may be involved. It was documented that the brain anatomy of soles is characterized by large olfactory and small optic lobes (Evans, 1937), and the olfactory sensors are highly developed (De Groot, 1969, 1971). It was demonstrated that the upper olfactory epithelium of soles is very sensitive to conspecific-derived odorants contained in different biological fluids, such as bile, intestinal fluid, and mucus (Velez et al., 2007, 2009). The authors suggested that intra-specific chemical communication is mainly mediated by the upper olfactory epithelium. So, it is plausible to hypothesize that this well-developed olfactory system may also be involved in mate recognition and selection, avoiding cross-breeding between the two Adriatic soles.

The increase of the condition factor between the end of summer and autumn could be explained by the favorable body conditions achieved to underpin the spawning period. The peak at the end of spring could indicate a recovery after the reproduction. In most fish species, energy reserves usually decrease in different organs and tissues, e.g., the muscle and liver, during breeding to support intensive generative synthesis and increase during the post-spawning and regeneration phases (Papiol et al., 2014). Thus, minimum condition values are measured during or immediately after spawning (Lloret et al., 2014). The trend of *S. solea* observed in this study differed somehow from that recently described in the same area (Bolognini et al., 2013). Indeed, the authors reported a decrease in the condition factor during winter, a gradual increment during spring, and the highest values in summer. It is worth noting that, despite the wide use of morphometric indices, these should be validated by correlating them with other physiological and biochemical parameters. Otherwise, morphological metrics should be considered as putative indicators of the condition (McPherson et al., 2011). Several studies found relationships between morphometric and other indices but, in some cases, they were weak or absent at all. So, to exhaustively explain the fish condition, it would be necessary to investigate parameters associated with functional condition and metabolic state, e.g., lipid components (Lloret et al., 2014).

The temporal trend of the hepatosomatic index corresponded to that of the condition factor, especially in females of both species and highlighted the higher energy investment of females than males during the reproductive period. The peaks before or during (autumn) and after (spring) reproduction indicated the energy investment in oogenesis and the recovery after reproduction, respectively. Indeed, the liver is the organ responsible for lipogenesis and is important for energy storage in several benthic and demersal species (Lloret et al., 2014; Papiol et al., 2014). Moreover, in teleosts, this organ has a key role in oogenesis and ovarian development (Nicolas, 1999).

The length at sexual maturity was evaluated considering specimens collected one month before and during spawning activity (Murua et al., 2003; ICES, 2010). The parameter was calculated only for females of *S. solea* because of the problems with the macroscopic maturity staging of males and the scarcity of small specimens of *S. aegyptiaca*. The result corresponded to the estimates for females in the Adriatic Sea, such as 25 cm L (Vallisneri et al., 2000) and 25.8 cm L (MEDISEH, 2013), while in the eastern Mediterranean, lower values of total length, namely 15.2 cm in Turkey (Türkmen, 2003) and 19.6 cm in Egypt (Mehanna, 2014) were reported. The lowest sizes of males and females with maturing gonads were 18 cm and 23 cm, respectively. Maturing *S. aegyptiaca* were observed for males and females from 18 and 20 cm, respectively. The same values corresponded to L_{50} estimates in Abu-Kir Bay (Egypt), where mature individuals were observed at lengths smaller than 15 cm (Abdel-Aziz, 1994). In Port Said (Egypt), L_{50} estimates were 14 cm (males) and 15 cm (females) (Mehanna, 2007; Ahmed et al., 2010). In the southern Tunisian waters, higher values than in the Egyptian area were obtained, 22.3 cm for males and 23.2 cm for females. The smallest specimens with mature gonads had similar sizes to those in the Adriatic Sea, 17 cm for males and 20 cm for females (Khalifa et al., 2019). In all the study areas, males of both species reached maturity earlier than females.

Fecundity is an important parameter to investigate associated with the fish size to scale estimates of spawning stock biomass or spawner abundance to population egg production (Murua et al., 2003). In this study, the threshold of 200 µm (oocyte diameter) was selected (based on the measurements taken from the histological sections to distinguish previtellogenic and vitellogenic oocytes), the same or similar value set in other studies for both species (Venema, 1964, in Houghton et al., 1985; Piccinetti and Giovanardi, 1984; Abdel-Aziz, 1994; Vallisneri et al., 2001). The hiatus between the previtellogenic and vitellogenic oocytes was not observed, but other authors reported its absence in diameter frequency distributions (Horwood and Greer Walker, 1990; Abdel-Aziz, 1994). Within a species, fecundity may vary in space and time because of several external (photoperiod and temperature, habitat conditions, food availability, species exploitation, etc.) and internal (fish condition, size, age, etc.) factors (Murua et al., 2003). In the Adriatic basin, the range of total or relative fecundity of *S. aegyptiaca* was higher than the values calculated in Tunisia and Egypt. The maximum diameter of hydrated oocytes measured was like the value reported by Abdel-Aziz (1994) in Egypt (Table 2). The fecundity of *S. solea* was widely investigated in the northeastern Atlantic and in some cases in captive broodstock (Houghton et al., 1985, Devauchelle et al., 1987, Ofelio et al., 2020). Horwood and Greer Walker (1990) compared the estimates from different study areas. The fecundity of the *S. solea* of 35 cm (or 458 g) ranged between 183,000 and 387,000 oocytes. In the Adriatic Sea, Piccinetti and Giovanardi (1984) estimated 150, 000 and 250,000 vitellogenic oocytes for females of 300 g and 400 g, respectively. Lower ranges were reported for the southeastern Mediterranean, except in the study of Mehanna (2014) in Egypt (Table 2). The differences found may be due to the method used and the size range of specimens analyzed.

In this study, fecundity estimates were similar between the two species, but the oocytes produced by *S. aegyptiaca* were significantly smaller than those of *S. solea*. Indeed, during sample processing, less voluminous ovaries were observed in *S. aegyptiaca* than in *S. solea* at the same length and gonadal development. Smaller eggs could mean a consequent smaller size of larvae and, eventually, a lower survival rate (Rijnsdorp and Vingerhoed, 1994; Hixon et al., 2014; Stuart et al., 2020; Koenigbauer and Höök, 2023), even if this is not always the rule (Ljubobratović et al., 2022). This characteristic may influence the reproductive success of the Egyptian sole in the Adriatic Sea.

5. Conclusion

In conclusion, gonadal development and reproductive strategy were similar between the two species. The main differences were observed in the cycle timing, with a shift between the spawning peaks, and oocyte size. Unfortunately, landings of *S. aegyptiaca* were scarce during the peak of reproductive activity because of the change of target species and fishing grounds by fishermen. Therefore, *ad hoc* sampling activity to collect further *S. aegyptiaca* during the spawning peak (winter-spring) will enable us to better describe the temporal variations of the reproductive cycle and could also unveil the presence of spawning aggregation areas. Future investigations could focus on the study of larval components to estimate the incidence of *S. aegyptiaca* and gather useful information about the fitness of the two congeneric species in the Adriatic basin.

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CRediT authorship contribution statement

Sabatini Laura: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Donato Fortunata**: Writing – review & editing, Methodology, Formal analysis. **La Mesa Mario**: Writing – review & editing, Methodology, Formal analysis. **Fanelli Emanuela**: Writing – review & editing, Conceptualization. **Scarcella Giuseppe**: Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Table 2
Fecundity estimates for Egyptian sole, *Solea aegyptiaca* (SA), and common sole, *S. solea* (SS), in the southeastern Mediterranean. F = potential fecundity; L = total length; S.D. = standard deviation.

	Mean *F* (S.D.)	*F* range	Hydrated max diameter (µm)	*L* range (cm)	Threshold (µm)	Reference and Locality
SA	70,980 (± 10,852)	14,250–125,110	1240	19.0–32.0	200	Abdel-Aziz (1994) Egypt
	-	9898–39,505	-	15.8–30.5	-	Ahmed et al. (2010) Egypt
	33,020 (± 5239)	14,160–62,700	-	16.5–30.7	-	Khalifa et al. (2019) Tunisia
SS	-	270,000–1200,000	-	-	-	Mehanna (2014) Egypt
	-	34,995–117,501	1200	22.2–32.9	-	Assem et al. (2019) Egypt
	-	13,924–341,479	-	19.7–30.4	120	Cerim and Ateş (2019) Turkey

Declaration of Competing Interest

All authors declare that no conflict of interest exists.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2025.107817.

Data availability statement

Data will be made available on request.

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