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The potential reproductive contribution of Mediterranean migrating eels to the *Anguilla anguilla* stock

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The European eel is a highly migratory fish. After the reproduction in the Sargasso Sea early larval-stages start a passive ocean migration towards European and Mediterranean continental waters. After several years as yellow eels, mature adults change to silver stage and then start their return trip. The trajectory of their backward migration is unknown, because of low probability of capturing migrating individuals, having this capture never been reported in the Mediterranean. Recently, 8 silver eels were collected in the Strait of Sicily. Using literature information about possible individual route and speed, their geographical position was projected up to the spawning site during reproductive season. Despite using optimal and continuous migration swimming speed, none of the specimens may have been able to reach the Sargasso Sea in time for mating. Subsequently, to identify putative Mediterranean areas from which eels could have been reaching the spawning grounds on time, a backward scenario was postulated using the previous scientific assumptions. Our results suggests that just a small quota of Mediterranean silver males successfully reaches the Sargasso area, and only females from the westernmost and central parts of the basin could be able to fruitfully pond their eggs during the supposed spawning period.

The European eel (*Anguilla anguilla* L., 1758) is a catadromous species whose life cycle is considered unique due to the amplitude of the spawning migration. Males mature at a smaller size than females (*i.e.*, at 30–45 cm and 80–180 g *versus* 60–80 cm and 400–1000 g¹). Silver eels (adult life-stage) migrate in autumn from European and North African coastal and inland waters to the Sargasso Sea, where they spawn and die. The resulting larvae (the leptocephali) drift backward to the Atlantic and Mediterranean continental shelf, then metamorphose into glass eels and colonize coastal and inland waters. Older juveniles (yellow eels) undergo a second transformation to adulthood, and complete the life cycle by migrating back to the Sargasso Sea, where in late spring they complete the maturation processes, mate, spawn and then die^{2,3}.

The European eel represents an important fishery commodity within its distribution area. Presently, the species is seriously threatened, as inferred from both the sharp recruitment decline, which is now less than 10% of what was observed in the past decades⁴, and the resulting decrease of fishery yields⁵. In 2007, the European Union released the Council Regulation 1100/2007/EC, for the recovery of the European eel stock, imposing on each member country to adopt an Eel Management Plan (EMP). A crucial aspect of such plans is to ensure an escapement to sea of at least 40% of the pristine silver eel biomass, *i.e.*, when no human activities affected the fishing area or the eel stock.

Such coordinated management measures have been enforced assuming that, in general, the species is a single and large panmictic population: in fact, the most recent and comprehensive study reported a very low and non significant genetic differentiation between geographic areas across Europe and a total lack of genetic structuring among larvae of European eel collected in the Sargasso Sea⁶.

That notwithstanding, several authors do not agree, suggesting that Mediterranean individuals may have a significantly reduced role on the actual spawning stock than the European Atlantic ones^{7–9}. Since the probability



of offshore capture of migrating adults is an extremely rare event (few dozens have been captured in the last 100 years, and none in the Mediterranean)⁹, these hypotheses should be confirmed with the support of both field data and theoretical simulations.

GIS technology offers the possibility of combining biological and spatial information in order to investigate fish movement and habitat use. In analyzing fish migration, the most usual applications of this technique concern the possibility to treat and plot the large amount of data acquired from animal tags and utilize them to generate models for individual species or eventually the whole ecosystems^{10,11}. In addition some software (e.g., the Animal Movement Analyst Extension, AMAE¹² has been especially developed in order to integrate new tools in GIS programs allowing to analyzing tracking data with analysis tools for a large collection of animal movement.

Here, we report the outcomes of geographic simulations performed with GIS software, carried out on 8 adult eels captured offshore in the Strait of Sicily (central Mediterranean), in order to identify putative trajectories and final points of the reproductive migration and verifying if these specimens could have been reaching the spawning grounds on time. Using the most favorable parameters from literature about swimming speed, silver migration time and possible individual routes, our aim is to show if, virtually, all Mediterranean silver male and female eels could successfully reach the Sargasso area in time, or if only a quota of them is able to fruitfully pond their eggs.

Results

Migrating silver eel catches and “forwarded scenario”. For the first time, 8 silver eels (Tab. 1) were incidentally collected in 5 different occasions on the slopes of the Strait of Sicily (Fig. 1a), by deep-water commercial bottom trawlers that operate in one of the heaviest exploited areas of the world¹³. The specimens were caught in daylight with only 1-2 specimens per haul (lasting a few hours each).

The eels were determined morphologically and genetically as *Anguilla anguilla*; the Sr/Ca of the otoliths confirmed that they had lived in freshwater; their muscles still had 30% (dry matter) of lipid reserves¹³. The eels showed signs of advanced maturation, such as extremely high ocular indexes¹⁴ (Fig. 1b).

To assess whether these eels would have been able to join the Sargasso Sea to mate and spawn, their geographical position was projected “forwarded” up to April 1st (Fig. 2), the month when several oceanic campaigns collected the most part of youngest leptocephali^{2,15}, since the exact spawning site has not yet been discovered.

This scenario was built using literature data on speed (0.65 and 0.40 m s⁻¹ for females and males respectively, corresponding to 0.75 and 1.05 body length s⁻¹)^{16,17}, stamina, routes and currents and the resulting most favorable parameters (i.e., continuous swimming, shortest route, etc.). However, none of the Sicilian specimens could have reached the Sargasso Sea on time, missing the spawning site by 5 weeks or more (eel #1 F, 52 d; eel #2 F, 31 d; eel #3 F, 31 d; eel #8 M, 108 d) or - as in the case of eel #4, 5, 6, and 7, fished June 1st - they would have been able to reach the Sargasso Sea only in the next mating season, about 10 months later (Tab. 2).

Backward retrospective scenarios. The question remained whether any other Mediterranean eel could participate in the reproductive event. Therefore, two “backward” scenarios, respectively for silver males and silver females, were built using the previous assumptions and some other ancillary information (e.g., size, latitude, hydrology, meteorology, temperature, all linked to the migration starting time/place, and marine currents), to show the areas of the Mediterranean coasts from which eels could have been reaching the spawning grounds on time. Also, we considered a different optimum swimming speed for both sexes^{16,17}. Our retrospective scenario was built with the most favorable assumptions: geographical (direct, shortest routes), temporal (early start of the migration, presence of opposite sex, short hatching lapse), and physiological (continuous swimming, optimum swimming speed, etc.). The results were overlaid on a geographical map using ArcGIS 10 (ESRI. ArcGIS desktop: release 10. Redlands, CA: Environmental Systems Research Institute, 2011).

The retrospective scenarios, based on possible/virtual time of departure during the migration seasons (i.e., from inland water) and swimming speed (Fig. 3), reveal that just a small quota of silver males escaping from the Mediterranean (Alboran Sea) could reach the Sargasso area in time to join the breeding event. On the contrary, silver females, with a higher average cruising speed, cover the same distance to the Sargasso Sea in less time, and therefore may reach the spawning area, theoretically from all the Mediterranean coastal areas.

Discussion

The capture of 8 silver eels in deep waters of the Central Mediterranean Sea is a unique event and a fundamental occasion to unveil some aspects of the still unknown life-cycle of one of the most intricate migratory fish of the world.

Apart the unquestionable rarity, the specimens were captured in deep water between 275 and 660 m in daylight. Silver eels are supposed to undertake distinct diel vertical migrations in the water column, in order to travel in warm, shallower waters at night, and in the deeper, cooler, disphotic zone during daylight¹⁸. Several explanations have been proposed for these daily dives: silver eels may swim into cooler waters in order to delay gonad development until later in the migration¹⁸, and also as a consequence of a visual predator-avoidance strategy¹⁹. Since continued residence in low temperatures reduces their swimming efficiency, silver eels may require a daily ascent to warmer water to increase metabolic rate and muscle efficiency^{18,20,21}. Besides, Righton et al.²² recently affirmed that *Anguilla* spp. muscle works well also on depths, since low temperature and high pressure favor aerobic energy production, thus reducing cost of swimming and improving the migratory efficiency.

All sampled individuals yielded a large amount of lipids in the muscular tissues (ca. 30%), a characteristic that in silver eels is crucial for completing the reproductive migration, not only for sustaining long-distance swimming, but also for gonad development and gametes production. In fact, silver eels should have at least 20% (out of total weight) of fat to migrate and reproduce successfully²³.

Simulations indicate that none of the captured silver eels (“forwarded” scenario) and only a very low percentage of silver males

Table 1 | Collection and morphological data of 8 European eels captured by trawl hauling in the Strait of Sicily (central Mediterranean) deep waters

	eel 1	eel 2	eel 3	eel 4	eel 5	eel 6	eel 7	eel 8
Total length (mm)	605	505	510	375	395	520	675	362
Weight (g)	384.4	204.8	258.3	78.9	106.9	205.5	646.9	97.2
Ocular index (%)	6.36	7.62	8.66	10.26	9.74	8.50	11.64	10.60
Sex	F	F	F	M	M	F	F	M
Age (yr; thin section)	4	9	6	6	n.a.	4	11	5
Average depth (m)	275	480	480	660	660	640	640	455

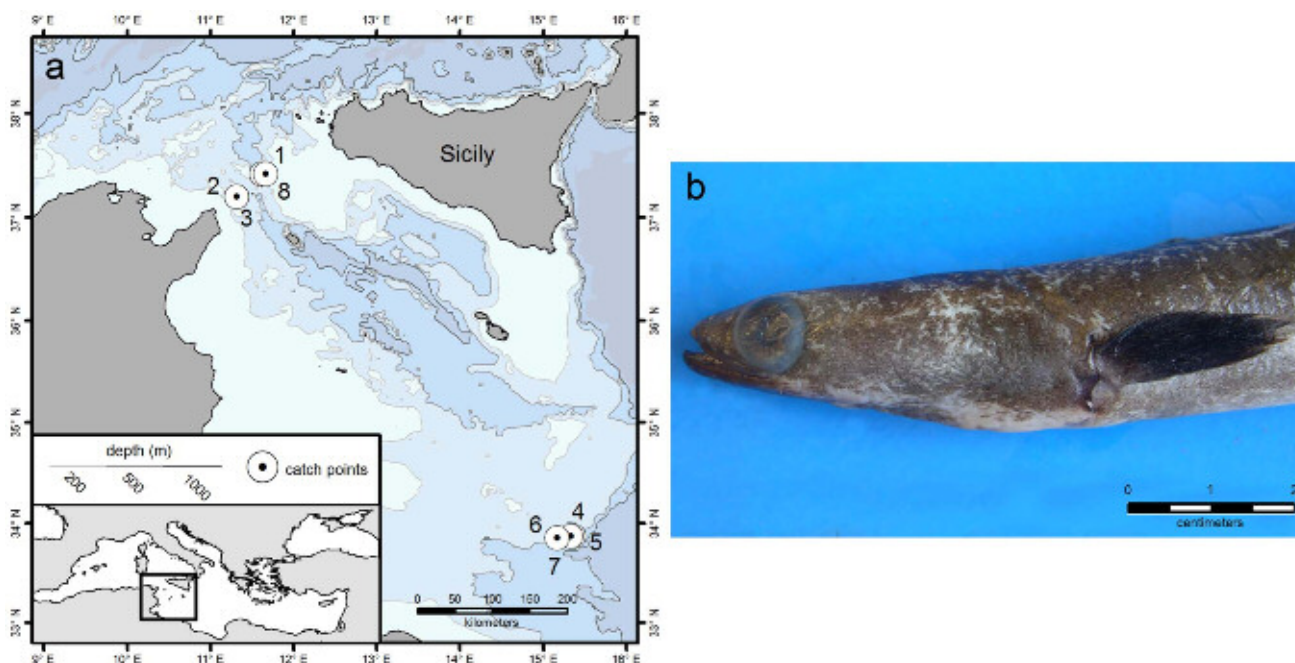


Figure 1 | The Strait of Sicily, with the catch location of deep-water migrating European eels (a), in stage of advanced maturation as shown by the head and eye of eel #4 ♀ (b). The map was drawn using the software ArcGIS Desktop: Release 10.

from the Mediterranean coasts (“backward retrospective” scenario) could reach the spawning area in due time. On the other hand, all female silver eels - even from the easternmost part of the basin - may be able to spawn, on condition that they do not delay up to November the beginning of migration.

In order to synchronize the arrival at a hypothetical breeding ground and maximize reproductive success, males, which have a slower swimming speed, should leave earlier²², or travel a shorter route to fertilize the eggs. However, the migrating time for silver eels to leave the continental domain and start the marine catadromic journey depends on many triggering factors²⁴: the season, the lunar phase, the decreasing temperature, the rain amount and the river water flow. Apart from possible exceptions, the leaving time goes from October to February, with in general a strong peak in November²⁵. The ample temporal “tail” may be due to the actual starting geographical location, which could be far upstream of long, undammed rivers (e.g., the Rhône, the Po, the lower Nile). Furthermore, migration of silver males and females from continental

waters starts in general simultaneously¹, and the postulated differential migratory start, does not result - at least at Mediterranean latitudes - from data on traditional fisheries landings, where males and females are contemporarily fished²⁶. Differently from the Atlantic and north European countries²⁷, weather conditions postpone the start of the silver eels migration until winter time in the whole Mediterranean basin, especially in eastern areas (A. Goda, Fish Nutrition Laboratory, National Institute of Oceanography and Fisheries, Egypt, personal communication 2011; E. Genc, Department of Fisheries and Aquaculture, Ankara University, Turkey, personal communication 2011). This phenomenon would make ineffective the journey of the majority of Mediterranean silver males (along with eastern females), which may need to leave the shores not later than November. In fact, the specimens caught in June in the Strait of Sicily are probably not stray eels, but may belong to such late, “useless” group.

The most recent sampling campaign in the Atlantic looking for the smallest European eel larvae proved that silver eel spawning period

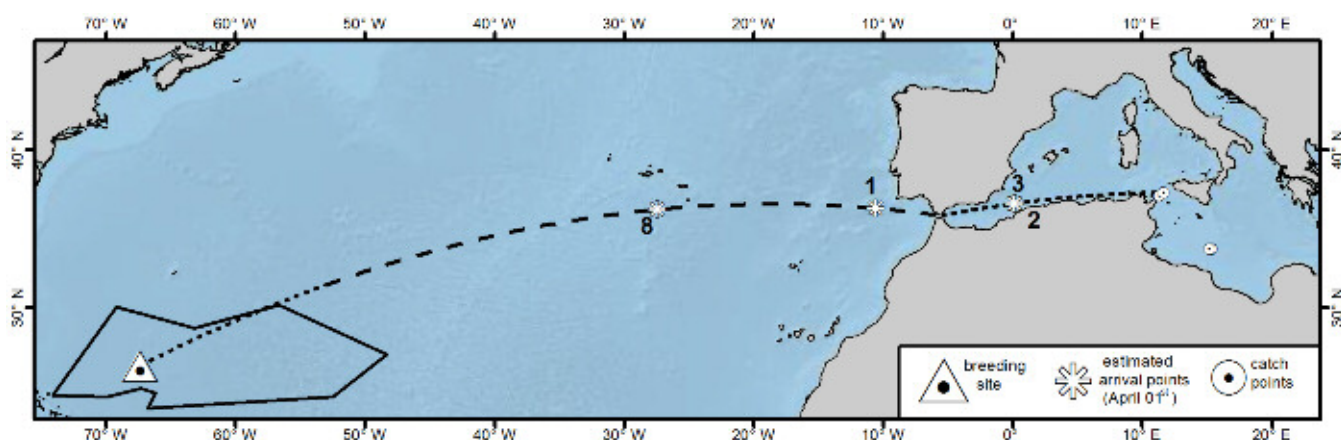


Figure 2 | The putative location of the European eels caught in the Strait of Sicily at the forwarded date of April 1st, the time limit for successful spawning. Individual routes, that are calculated as the shortest possible, are not straight since reflecting the globe curvature. The maps were drawn using the software ArcGIS Desktop: Release 10.



Table 2 | Body lengths, swimming speeds and distances potentially covered by each individual in their migration in the period starting from their catch date to April 1st. For specimen caught on June 1st, migration time and distance have not been computed, since putative arrival date at spawning ground is even two months before their sampling

	eel 1	eel 2	eel 3	eel 4	eel 5	eel 6	eel 7	eel 8
Total length (mm)	605	505	510	375	395	520	675	362
Swimming speed (m s ⁻¹)	0.454	0.379	0.383	0.395	0.416	0.390	0.506	0.381
Catch date	February 9 th	March 1 st	March 1 st	June 1 st	June 1 st	June 1 st	June 1 st	December 14 th
Arrival date	April 1 st	April 1 st	April 1 st	April 1 st	April 1 st	April 1 st	April 1 st	April 1 st
Migration time (d)	52	31	31	n.a.	n.a.	n.a.	n.a.	108
Distance (km)	2039	1014	1024	n.a.	n.a.	n.a.	n.a.	3557

occurs from March through May, with a peak of reproductive activity in April². Although Schmidt collected intensively in the spawning area in June and July 1920 and June 1922 and captured²⁸ - among a few thousand larger specimens - also about 85 leptocephali <10 mm long in June and another 4 in July, there is little evidence from field studies of spawning in the latter half of the year.

Maes et al.²⁹ found significant genetic differentiation between early and late glass eels recruiting in a Mediterranean coastal lagoon in France. They suggested several alternative hypotheses for such temporal genetic differentiation, among which the existence of two dis-

crete spawning events in the Sargasso Sea. This scenario was depicted also by Wang and Tzeng³⁰ who, studying otoliths of European glass eels, reported a protracted spawning period, but peaking earlier, in January. However, temporal genetic differences could be the consequence of the offspring from two different reproductive years too. Another possible scenario, inducing such patterns, is genetic patchiness³¹⁻³³, at present the most likely hypothesis: the combined effect of an effectively restricted number of parents and differential seasonal survival rates of offspring during the long transoceanic migration that is affected also by fluctuating oceanic condition.

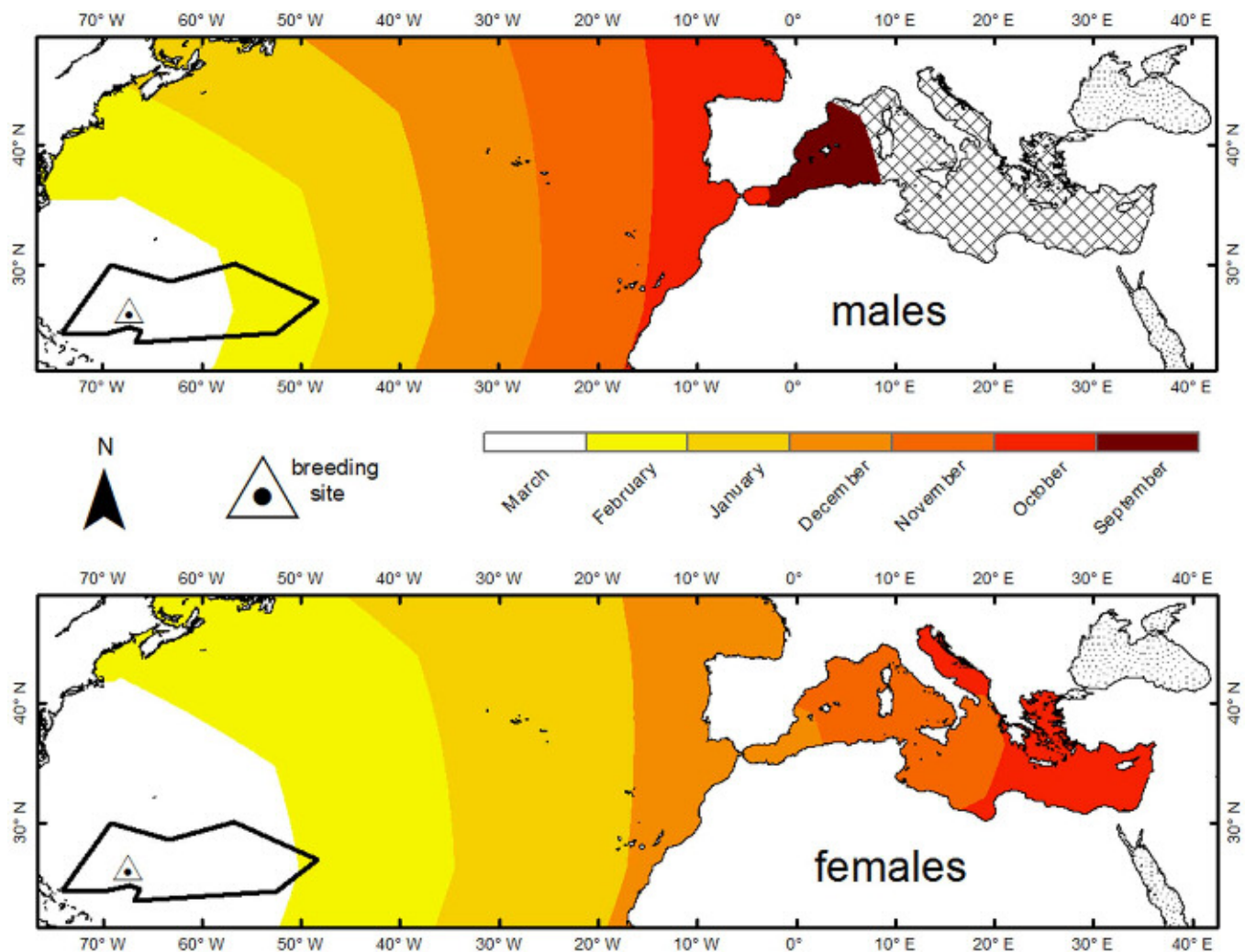


Figure 3 | Areas from where, depending on the starting period of the catadromic journey, European eels could reach the spawning grounds on time for successful reproduction; the two scenarios (upper panel, ♂; lower panel, ♀) reflect the differences in speed between sexes. The polygon encompasses the area where small leptocephali [<10 mm] have been collected; eels do not live in Black Sea nor in Red Sea areas. The maps were drawn using the software ArcGIS Desktop: Release 10.



Concerning the duration of their travel towards the Sargasso Sea, endurance and stamina of the migrating eels are critical factors for successfully reaching the breeding place. Their journey was previously believed to last around 6 months, while more recently the estimation fell to just 4 months¹⁶. In fact, there are no field data at present concerning the duration of the oceanic catadromic migration. However, since silver eels cease feeding when starting their migration¹, they must rely on their energy storage for both swimming and reproduction. In this scenario, it seems probable that they should reach the spawning ground during the first possible mating window, to optimize the use of reserves accumulated during the continental life. An alternative scenario, which seems not valid for the Mediterranean, is that the eels would reach the Sargasso Sea in the following mating season, with a traveling period greater than one year. This hypothesis, of a migrating journey towards the Sargasso Sea longer than one winter, may be used to offer a chance of reproduction also to silver eels from the Mediterranean Sea. Without this assumption, individuals would not be able to reach the reproductive site in the spring. Although it cannot be neither confirmed nor rejected by (inexistent) experimental/field data, a scenario where a migration period longer than the 6–9 months is commonplace seems difficult to accept. Since silver eels cease feeding during the catadromic migration³⁴, in this last situation the limited energy storage of eels must provide not only fuel for swimming and for gonad maturation, but also for routine metabolism³⁵. Given that fat levels in silver eels from many in Mediterranean environments^{36–38} are similar to what estimated to be just enough to successfully complete the reproduction²³, this fact seems inconsistent with the possibility of spending more than one year at sea without any energy intake.

Moreover, gonad development must be delayed or even stopped while waiting for the next spawning period. In fact, silver eels descending to deeper waters during the oceanic migration might delay gonad development thanks to temperatures lower than 11 °C^{18,39}. On the contrary, the Mediterranean temperatures, unlike those of the Atlantic Ocean, remain largely uniform at around 12.5–14.5 °C at all depths below the thermocline (200 m depth⁴⁰), being thus ineffective for delaying maturation.

It is still uncertain whether eels swim continuously during their migration, whether they rest regularly or whether they profit from the oceanic currents⁴¹. There are contrasting data on the daily activity, with some tracking experiments showing uninterrupted swimming⁴², while other authors observed in shallow waters, just after leaving the coastal environments, only nocturnal swimming, with the animals resting on the bottom at daylight^{43,44}.

About the use of currents to promote migration by increasing swimming speeds, it should be considered that no substantial westward drift exist in the Mediterranean. In the Atlantic two migration routes to the Sargasso Sea have been proposed: a northern migration route *via* the central Atlantic Ocean⁴⁵, which is the same hypothesis used in the present research, and a southern route *via* the Azores Current⁴². In fairness, it should be stated that present knowledge about the Atlantic Ocean phase of the migration is very limited⁴⁶. Although *A. anguilla* could potentially make use of the North Equatorial Current (NEC) to facilitate long distance swimming^{18,47} we decide to consider a northern migration route *via* the central Atlantic Ocean⁴⁵ because, according to our aim of understanding the timing of arrival of silver eels, the use of a southern route *via* the NEC would increase the migration distance⁴¹, from Gibraltar to the spawning point, of about 30%, nullifying any possible, and difficult to calculate, advantage arising from entering in the NEC.

In conclusion, the annual arrival of larval and glass eels on every coast in the distribution range is proof enough that European eels do overcome all the challenges of their life history, such as synchronicity of arrival and spawning success. That notwithstanding, according to our results, part of the Mediterranean stock may be unable to contribute to the panmixia. This would indicate that *A. anguilla* distri-

bution may follow a modified single-species Tucker's mechanism, based on swimming energetic arguments⁴⁸. Accordingly, only a portion of the entire eel population is responsible for replenishing the whole distribution area, in agreement with results showing no evidence for a genetic bottleneck in the European eel. A stable, effective population size of around 3000–12000 individuals, as calculated through forward-time simulations, might be sufficient to sustain the observed levels of genetic diversity⁴⁹.

As described above in details, our results come from GIS-based theoretic scenarios, rather than mathematical models, which allow, for the first time, to figure out the fate of male and female silver eels leaving the Mediterranean coastal environments towards their reproductive ground in the Sargasso sea. The data and assumption considered for building these scenarios are quite permissive (early start of silver migration, continuous optimal swimming speed, direct routes) in order to adopt a precautionary approach; nonetheless, results point out clues that should be considered not only for further studies of basic biology, but also in the perspective of the complex international framework of policies striving to protect and manage this declining resource. In fact such indications should be confirmed in the near future by additional biological data from both larvae and silver eels catches during the oceanic migration in order to better understand the complex life cycle of this species.

Methods

Biological material. Since the present work did not rely on actual animals, there was no need of approved guidelines for animal welfare by any institutional and/or licensing committee.

All the eels were caught in the Strait of Sicily by commercial bottom trawlers targeting red shrimps, in daylight on grounds 300–650 m deep; fishermen froze the animals without any precise protocol and brought them to the CNR lab in Mazara del Vallo (southern Sicily).

Eels were measured for biometric analyses, sexed histologically, X rayed for vertebral count, and their otoliths extracted. Otoliths were ground to thin sections, and age was assessed with a light microscope. In order to verify the freshwater history, the sections were also microanalysed for Sr and Ca with quantitative X ray mapping, using a Cambridge S360 SEM connected to an Inca Energy 200 Energy Dispersive Spectrometer. Samples of flesh were analysed for their proximate composition; lipids were extracted and the fatty acids profile determined by gas chromatography. Total genomic DNA was extracted from other flesh samples, and mtDNA (cytochrome b) sequences were amplified via PCR; amplification products were finally sequenced and compared with GenBank records.

Mapping. Simulating individual movements on the seascape requires a model which allows both to consider the presence of terrain obstacles and to minimize the distortion introduced by the use of projected spatial data in calculating distances on a geoid surface.

Based on these assumptions we choose a network-based approach in combination with the use of geodetic primitives. All the procedures were implemented using ESRI ArcGIS (rel. 10.1) tools.

Network analysis is a widely applied framework in geography, information technology and computer science. It is primarily concerned with the minimization of transportation costs in the movement of people and goods⁵⁰ but more recently it was also used to analyze ecological processes concerned with the analysis of potential pathways and connectedness^{51,52}.

According to graph theory⁵³, a network is a lattice consisting of a set of objects, called nodes, with certain pairs of these objects connected by links, called edges⁵⁴. The links might be of various kinds, representing a logical or a geographical connection between two nodes; commonly in the geographical case they represent the potential movement of an organism among the network and they are associated with an attribute (impedance) representing the cost of passing from a node to another.

A common problem in network analysis is to find the shortest route between two points. The classical solution is performed by using the Dijkstra's⁵⁵ algorithm. To determine the best path, the algorithm scans the road network for all nodes adjacent to the origin node. All links to these nodes are assessed, and the lowest cumulative impedance from the origin is selected at each node until the destination node is reached. When working with networks concentrated in a relatively small area and with an appropriate projected coordinate system that minimizes distance distortion, the Euclidean distance can be used as impedance in order to express the shortest route from a point to another, but in the case of long distances the overall route deviates significantly from the shortest path. In order to minimize this deviation the edges used to build the network need to be geodetic vectors representing the shortest line linking two points in the space considering earth curvature⁵⁶.

In our case we built a network dataset representing the area of Mediterranean and Sargasso Seas. Initially a grid of points, placed at different distances ranges from 5 to 15 km, was positioned in the geographical space representing the study area, created



by masking all land features obtained from a world vector map⁵⁷; then each point was connected with its 8 neighbours using geodetic vectors and for each of them the corresponding geodetic distance was calculated using Vincenty's equations⁵⁸.

Forwarded eels scenario. The first GIS scenario (Fig. 2) was built in order to identify the potential geographic positions in the study area where each eel, starting from its catch point, could have been found during the migration to the spawning site.

The calculation has been performed by first identifying for each individual the shortest route from the original positions to the spawning point, and then calculating the accumulated geodetic distance that it could cover during the considered time period according to its sex, size and swimming velocity.

All the paths were determined by using the network analyst tool (NAT) available in ArcGIS and then smoothed in order to assign a more realistic shape. The starting points for each path are the catch locations and the arrival point the site where the aggregations of larvae appear as the densest (26°N, 67.5°W)⁵⁹. That location was identified during several scientific cruises over the wide area of the Sargasso Sea (23–30°N, 48–74°W) since 1911⁶⁰.

Our computations took into account the date of the April 1st as theoretical arrival date of silver eels to the spawning site. Evidence from sampling in the Sargasso Sea for leptocephali of European eels, that has occurred over different seasons, decisively suggests a restricted spawning season, to a few months mainly in late winter and spring^{2,3,28,58–61}.

Eels geographic positions were “forwarded” up to April 1st, using the shortest routes from each catch point to the spawning site and considering different continuous cruise swimming speed for each sexes. Experimental studies on swimming performance and energy consumption simulating the migration in swim tunnel (Blazka type 127-l) established that *A. anguilla* is very efficient endurance swimmer by estimating the optimum swimming speed, i.e., the speed at which the cost of transport ($\text{mg O}_2 \text{ kg}^{-1} \text{ km}^{-1}$) is minimal, finding a mean value for wild female silver eels around 0.65 m s^{-1} (or, equivalently, 0.75 body length s^{-1})¹⁶ and for farmed silver males of 0.40 m s^{-1} (or, equivalently, 1.10 body length s^{-1})¹⁷. These values, at least for females, agrees with the sustained swimming speed cruise currently observed in field tracking studies on migrating females^{1,19,20,41,62}, in particular when considering the fastest measured records (even considering that swimming speeds might have been impaired by the tag burden)^{63,64}.

Backward retrospective scenarios. The second GIS scenario (Fig. 3) was a “backward” displacement computation, which identified the corresponding potential period of starting migration time from each zone in the study area of the southern distribution area of *A. anguilla*. The computation performed assumes that mature individuals should arrive in the spawning site (26°N, 67.5°W) at the Sargasso Sea on the following April 1st, with respect of the time of departure from Mediterranean coastal environments. Also in this case, two different swimming speeds were considered, using published speed data (0.65 and 0.40 m s^{-1} for females and males, respectively)^{16,17}.

The calculation was performed in two steps. Initially a distance map of the study area was produced by generating a raster layer where each cell represents the geodetic distance from the spawning site. Geodetic distances were computed using iteratively NAT tools, considering as starting point the spawning site and as arrivals a grid of points spaced from 30 to 45 km. In this way we obtained about 12,000 routes representing the optimal paths from the spawning site to a series of location equally distributed on the study area. For each route the accumulated geodetic distance was calculated and the obtained value was assigned to the corresponding point. Finally, the point layer was converted in raster format by interpolating point values with a spline algorithm.

The distance map was then used to produce the two backward scenarios, respectively for males and females, by calculating for each cell the corresponding period of migration considering the two different optimum swimming speeds. Finally, the two raster maps were classified in monthly ranges, showing in the study area the time period when an eel should have been starting its migration in order to reach the spawning point on April 1st.

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Author contributions

All the authors wrote the main manuscript text. F.C., C.C., E.C. and M.L.B. analyzed data. M.L.B., S.R. and E.C. prepared figure 1, E.C. prepared figure 2 and 3. F.C., J.A., M.L.B., F.A., S.R., and C.C. reviewed the manuscript.

Additional information

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