Universitat de Girona

Variability in the health status and reproductive traits of European sardine stocks in the Mediterranean. Implications for fishery management

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Doctoral Thesis

MARTA CABALLERO-HUERTAS

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DOCTORAL THESIS

Variability in the health status and reproductive traits of European sardine stocks in the Mediterranean. Implications for fishery management

Marta Caballero-Huertas

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Doctoral Programme in the Environment

Ph.D. Advisors

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Thesis submitted in fulfilment of the requirements to obtain the Ph.D. Degree at the University of Girona

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Universitat de Girona

Dr. Marta Muñoz Frigola from the Institute of Aquatic Ecology and Dr. Jordi Viñas de Puig from the Laboratory of Genetic Ichthyology, both from the University of Girona, as supervisors of this Doctoral Thesis **declare:**

That the Doctoral Thesis entitled **"Variability in the health status and reproductive traits of European sardine stocks in the Mediterranean. Implications for fishery management"** presented by **Marta Caballero Huertas** in the pursuance of the Ph.D. Degree has been completed under our supervision and meets the conditions required to opt for an International Doctorate.

In witness thereof, we hereby sign this document.

Dr. Marta Muñoz Frigola

Dr. Jordi Viñas de Puig

Girona, April 2023

"The future is in the hands of those who explore... and from all the beauty they discover while crossing perpetually receding frontiers, they develop for nature and for humankind an infinite love."

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Supplementary materials

List of abbreviations

AEMET: State Meteorological Agency

Alb: Alboran Sea

Alb. C: Alboran Sea, Coastal area

Alb. O: Alboran Sea, Open Sea

ANOVA: One-way analysis of variance

CaM-4: Calmodulin-4

CG2: Cabo de Gata inshore

CG4: Cabo de Gata offshore

Chl: chlorophyll concentration

D: Dorsal

ddRADseq: Double digest restriction-site associated DNA

df: Degrees of freedom

DNA: Deoxyribonucleic acid

EPIC-PCR: Exon-primed intron-crossing PCR

f: female

FAO: Food and Agriculture Organization of the United Nations

F_{ST}: Fixation index

G: Guadiana River mouth

GC. C: Gulf of Cádiz, Coastal area

GC. O: Gulf of Cádiz, Open Sea

GD: Gualdalquivir River offshore

GFCM: General Fisheries Commission for the Mediterranean

GQ: in front of the Guadalquivir River mouth

GSA: Geographical subareas

GSA 1: Northern Alboran Sea

GSA 2: Alboran Island

GSA 3: Southern Alboran Sea

GSA 6: Northern Spain

GSA 7: Gulf of Lions

GSA 16: South of Sicily

GSA 17: Northern Adriatic

GSA 18: Southern Adriatic Sea

GSA 22: Aegean Sea

GSI: Gonadosomatic index

h: Haplotype diversity for mitochondrial markers

He: Expected heterozygosity

Ho: Observed heterozygosity for nuclear markers

$\bar{H}_S$: Subpopulation heterozygosity

HSI: Hepatosomatic index

$\bar{H}_T$: Average total heterozygosity

ITS: Internal transcribed spacer

Kn: Relative condition factor

L_T: Total length

m: male

M: Mouth of Rivers Tinto and Odiel

MLS: Minimum landing size

mtDNA: Mitochondrial DNA

mtDNA *cox2*: Mitochondrial cytochrome c oxidase subunit II

mtDNA-RFLP: Mitochondrial DNA Restriction Fragment Length Polymorphism

N: Total number of samples

NA: Data not reported/No data available

N_c: Census size

N_{coll}: Number of collected ascaridoid larvae

N_e: Effective population size

N_{id}: Number of identified larvae

NS: Not statistically significant

OPFish: Ocean productivity available to fish

P: Precipitation rates

P2: Cabopino inshore

P4: Cabopino offshore

P-A: Portimao-Albufeira

PCB: Polychlorinated biphenyl

PCR: Polymerase Chain Reaction

POR: Southern Portugal

POR-GC: Southern Portugal-Gulf of Cádiz

SM: Cape Santa María

SNPs: Single-nucleotide polymorphism

SP: Sancti Petri

SPF: Small pelagic fish

SV: Cape San Vicente

SST: Sea Surface Temperature

T: Trafalgar

Ux: West-east wind component

Uy: South-north wind component

V: Ventral

V2: Vélez inshore

V4: Vélez offshore

Vi: Stomach vacuity index

W_E: Eviscerated weight

W_G: Gonad weight

W_L: Liver weight

W_T: Total body weight

Summary

Small pelagic fish play an important ecological role mainly due to their contribution in transferring energy from low to higher trophic levels. Likewise, the capture of these species is in many cases an important nutritional and economic source for many nations. In these respects, one of the most relevant pelagic species is the European sardine (*Sardina pilchardus*) throughout its distributional range, in the North and Central-Eastern Atlantic, from the North Sea to the Senegalese coast, including the Mediterranean Sea, in which an alarming health status of the stocks has been registered during the last decades. Despite its importance, there are still many unanswered questions that try to explain its variability in the state of health along the different areas where it is found. Furthermore, and due to the decline in captures, biomass and body condition (i.e., individual energy reservoirs) of the resource, it has been suggested that the effect of global change, overfishing, pollution or parasitism, and/or the interaction among factors could be behind it. This thesis has been enthusiastically carried out with the aim of shedding light on the current health status of the European sardine along the Mediterranean, highlighting the assessment of the state of condition (closely related to the annual reproductive cycle), as well as on determining the likely factors that shape its spatial and, potentially, temporal variability in this regard. The confluence of genetic, physiological and ecological information has been the central axis of this work, since it is essential to approach the study of this and other fishery resources from a holistic manner, especially if this information is to be applied to the field of fisheries administration and management.

After the combination of different methodologies, standing out the evaluation of the condition of sardine individuals through lipid estimation and the calculation of indices, the study of the gonads, and the detailed parasitic inspection and application of genetic techniques for the identification of nematodes, results indicated a clear spatial variability along the Mediterranean coast and considering individuals from the nearby Atlantic waters in sardine's body condition, reproduction (investment, phenology, strategy, and gonad alterations), and nematode parasitism throughout the annual cycle. These studied features are interconnected, and depend on key environmental variables, especially, temperature, productivity (closely linked to proximity to rivers, wind, the upwelling of deep waters, the physical barriers of the coast, etc.), and those characteristics of the history of life of the stocks that have been shaped through the generations and, consequently, that are linked to genetic differences. In fact, it was concluded that the accessibility to the resources in the environment modulates the reproductive strategy of European sardine, and that the liver presents notable differences linked to the characteristics of the stock (i.e., larger livers in

the highly productive Northern Adriatic, which is defined as an independent genetic unit). Additionally, the drop in the local temperature in the Alboran, located in the Atlantic-Mediterranean transition, helped by the inflow of Atlantic waters in the Mediterranean and other oceanographic conditions that favour nursery grounds trigger the migration of Atlantic individuals to the Mediterranean area, verified by the condition and the reproductive phenology on both sides of the Strait of Gibraltar. In addition, the species and parasite load of the three nematodes infecting sardine (*Hysterothylacium aduncum*, as the most prevalent, and *Anisakis pegreffii* and *A. simplex* (s.s.)) diverge between stocks, being absent in the Alboran and the Northern Aegean. When exploring the parasitic load at seasonal level, almost none difference between infected and non-infected individuals were observed in condition parameters, as well as no relationship between the abundance of parasites and fish energetic status was obtained, but there was a clear link to the reproductive stage. Moreover, three-lobed testes were found for the first time in sardines from three Mediterranean locations (Northern Spain, Northern Adriatic and Northern Aegean Sea), although its aetiology is still to be determined. On the other hand, the review of the knowledge status of sardine genetic stocks has revealed large estimates of genetic diversity, a spatial mismatch between the sardine's populations and the managed stocks currently defined, and an uneven study effort along sardine's distribution regarding genetic structuring.

These results are crucial to understand key aspects in the biology of sardine, which may help to project the future of the species under the current environmental scenario. This thesis is not only an approach to the integration of aspects of the ecology of the European sardine, but also a manual for understanding the current status of this species and providing recommendations for future management lines.

Resum

Els peixos pelàgics de mida petita tenen un paper ecològic important sobretot per la seva contribució en la transferència d'energia de nivells tròfics baixos a més alts. Així mateix, la captura d'aquestes espècies és en molts casos una important font nutricional i econòmica per a moltes nacions. En aquests aspectes, una de les espècies pelàgiques més rellevants és la sardina europea (*Sardina pilchardus*) en tota la seva distribució, a l'Atlàntic nord i centre-oriental, des del mar del Nord fins a la costa senegalesa, passant per la Mediterrània, en la qual s'ha registrat un estat de salut alarmant dels *stocks* durant les últimes dècades. Malgrat la seva importància, encara queden moltes preguntes sense resposta que intenten explicar la seva variabilitat a l'estat de salut al llarg de les diferents zones on es troba. A més, i a causa del descens de les captures, la biomassa i la condició (és a dir, els dipòsits d'energia dels individus) del recurs, es planteja que l'efecte del canvi global, la sobrepesca, la contaminació o el parasitisme, i/o la interacció entre factors podria estar al darrere. Aquesta tesi s'ha realitzat amb entusiasme amb l'objectiu de donar llum sobre l'estat de salut actual de la sardina europea al llarg del Mediterrani, destacant l'avaluació de la condició (estretament relacionat amb el cicle reproductiu anual), així com per a determinar els factors que configuren la seva variabilitat espacial i, potencialment, temporal en aquest aspecte. La confluència d'informació genètica, fisiològica i ecològica ha estat l'eix central d'aquest treball, ja que és fonamental abordar l'estudi d'aquest i d'altres recursos pesquers des d'una manera holística, sobretot si aquesta informació es vol aplicar a l'àmbit de l'administració i gestió pesquera.

Després de la combinació de diferents metodologies, destacant l'avaluació de la condició dels individus de sardina mitjançant l'estimació de lípids i el càlcul d'índexs, l'estudi de les gònades, i la inspecció detallada dels paràsits i aplicació de tècniques genètiques per a la identificació de nematodes, els resultats van indicar una clara variabilitat espacial al llarg de la costa mediterrània i tenint en compte els individus de les aigües atlàntiques properes en l'estat corporal de la sardina, la reproducció (inversió, fenologia, estratègia i alteracions de les gònades), i el parasitisme per nematodes a llarg del cicle anual. Aquests trets estudiats estan interconnectats, i depenen de variables ambientals clau, especialment, la temperatura, la productivitat (estretament vinculada a la proximitat als rius, el vent, la surgència d'aigües profundes, les barreres físiques de la costa, etc.), i aquelles característiques del història de vida de les poblacions que s'han anat configurant al llarg de les generacions i, conseqüentment, que estan lligades a diferències genètiques. De fet, es va concloure que l'accessibilitat als recursos del medi modula l'estratègia reproductiva de la sardina europea, i que el fetge presenta diferències notables lligades a les característiques

de l'estoc (és a dir, fetges més grans a l'altament productiu Adriàtic Nord, definit com una unitat genètica independent). A més, la caiguda de la temperatura local a l'Alborà, situat en la transició atlàntica-mediterrània, ajudada per l'entrada d'aigües atlàntiques a la Mediterrània i altres condicions oceanogràfiques que afavoreixen les zones de cria desencadenen la migració d'individus atlàntics cap a la zona mediterrània, comprovat mitjançant la condició i la fenologia reproductiva a banda i banda de l'estret de Gibraltar. A més, la càrrega de paràsits i les espècies dels tres nematodes que infecten la sardina (*Hysterothylacium aduncum*, com a més prevalent, i *Anisakis pegreffii* i *A. simplex* (s.s.)) divergeixen entre les poblacions, sent absents a l'Alboran i al nord de l'Egeu. Quan es va explorar la càrrega parasitària a nivell estacional, gairebé no es va observar cap diferència entre individus infectats i no infectats en els paràmetres de condició, així com no es va obtenir cap relació entre l'abundància de paràsits i l'estat energètic dels peixos, però hi va haver un vincle clar amb l'etapa reproductiva. A més, es van trobar per primera vegada testicles trilobulats en sardines de tres localitats mediterrànies (nord d'Espanya, nord de l'Adriàtic i nord del mar Egeu), tot i que encara està per determinar-ne l'etiologia. D'altra banda, la revisió de l'estat de coneixement dels estocs genètics de sardina ha revelat grans estimacions de diversitat genètica, un desajust espacial entre les poblacions de sardina i les poblacions gestionades actualment definides, i un esforç d'estudi desigual al llarg de la distribució de la sardina pel que fa a l'estructuració genètica.

Aquests resultats són crucials per entendre aspectes clau en la biologia de la sardina, que poden ajudar a projectar el futur de l'espècie sota l'escenari ambiental actual. Aquesta tesi no és només una aproximació a la integració d'aspectes de l'ecologia de la sardina europea, sinó també un manual per entendre l'estat actual d'aquesta espècie i aportar recomanacions per a futures línies de gestió.

Resumen

Los peces pelágicos de tamaño pequeño juegan un papel ecológico importante principalmente debido a su contribución en la transferencia de energía de los niveles tróficos bajos a los más altos. Asimismo, la captura de estas especies es en muchos casos una importante fuente nutricional y económica para muchas naciones. En estos aspectos, una de las especies pelágicas más relevantes es la sardina europea (*Sardina pilchardus*) en todo su rango de distribución, en el Atlántico Norte y Centro-Este, desde el Mar del Norte hasta la costa de Senegal, pasando por el Mediterráneo, en el que se ha registrado un alarmante estado de salud de los *stocks* durante las últimas décadas. A pesar de su importancia, aún quedan muchas preguntas sin respuesta que intentan explicar su variabilidad en el estado de salud a lo largo de las distintas zonas donde se encuentra. Además, y debido a la disminución de las capturas, la biomasa y la condición (es decir, depósitos de energía de los individuos) del recurso, se viene planteando que detrás podría estar el efecto del cambio global, la sobrepesca, la contaminación o el parasitismo, y/o la interacción entre factores. Esta tesis se ha realizado con entusiasmo con el objetivo de arrojar luz sobre el estado de salud actual de la sardina europea a lo largo del Mediterráneo, destacando la evaluación de la condición (muy relacionada con el ciclo reproductivo anual), así como para determinar los factores que configuran su variabilidad espacial y, potencialmente, temporal en este aspecto. La confluencia de información genética, fisiológica y ecológica ha sido el eje central de este trabajo, ya que es fundamental abordar el estudio de este y otros recursos pesqueros de manera holística, sobre todo si se quiere aplicar esta información al campo de la administración y gestión pesquera.

Tras la combinación de diferentes metodologías, destacándose la evaluación de la condición de individuos de sardina a través de la estimación de lípidos y el cálculo de índices, el estudio de las gónadas, y la inspección parasitaria detallada y aplicación de técnicas genéticas para la identificación de nematodos, los resultados indican una clara variabilidad espacial a lo largo de la costa mediterránea y considerando individuos de las aguas atlánticas cercanas en la condición corporal, la reproducción de la sardina (inversión, fenología, estrategia y alteraciones gonadales), y el parasitismo por nematodos a lo largo del ciclo anual. Estos rasgos estudiados están interconectados y dependen de variables ambientales clave, en especial, la temperatura, la productividad (estrechamente ligada a la proximidad de los ríos, el viento, el afloramiento de aguas profundas, las barreras físicas de la costa, etc.), y aquellas características de la historia de vida de los *stocks* que se han ido configurando a través de las generaciones y, consecuentemente, están ligadas a diferencias genéticas. De hecho, se ha concluido que la accesibilidad a los recursos en el medio modula

la estrategia reproductiva de la sardina europea, y que el hígado presenta diferencias notables ligadas a las características de la población (es decir, hígados más grandes en el altamente productivo Adriático norte, definido como una unidad genética independiente). Además, el descenso de la temperatura local en el Alborán, situado en la transición Atlántico-Mediterráneo, ayudado por la entrada de aguas atlánticas en el Mediterráneo y otras condiciones oceanográficas que favorecen las zonas de cría desencadenan la migración de individuos atlánticos hacia el área mediterránea, constatado por la condición y la fenología reproductiva a ambos lados del Estrecho de Gibraltar. Además, las especies y la carga parasitaria de los tres nematodos que infectan a la sardina (*Hysterothylacium aduncum*, como el más prevalente, y *Anisakis pegreffii* y *A. simplex* (s.s.)) divergen entre las poblaciones, estando ausentes en Alborán y el Egeo septentrional. Al explorar la carga parasitaria a nivel estacional, casi no se observaron diferencias entre los individuos infectados y no infectados en los parámetros de condición, así como tampoco se obtuvo una relación entre la abundancia de parásitos y el estado energético de los peces, pero hubo un vínculo claro con la etapa reproductiva. Además, se encontraron por primera vez testículos trilobulados en sardinas de tres localidades mediterráneas (norte de España, norte del Adriático y norte del mar Egeo), aunque su etiología está aún por determinar. Por otro lado, la revisión del estado del conocimiento de los *stocks* genéticos de sardina ha revelado grandes estimaciones de diversidad genética, un desajuste espacial entre las poblaciones de sardina y los *stocks* gestionados actualmente definidos, y un esfuerzo de estudio desigual a lo largo de la distribución de sardina con respecto a la estructura genética.

Estos resultados son cruciales para comprender aspectos clave en la biología de la sardina, que pueden ayudar a proyectar el futuro de la especie en el escenario ambiental actual. Esta tesis no es solo una aproximación a la integración de aspectos de la ecología de la sardina europea, sino también un manual para entender el estado actual de esta especie y aportar recomendaciones para futuras líneas de gestión.

General introduction

1. The European sardine in marine ecosystems: ecological and economical values

Small Pelagic Fish (SPF) are present in many regions of the world's oceans. Their habitats include areas with coastal and oceanic upwelling and freshwater influence and can be characterised by both geography (properties of the coast and bottom) and hydrography (properties of the water) (Checkley et al., 2009). As forage fish, which feed primarily on plankton (Blaxter & Hunter, 1982), they play a leading role in marine ecosystems transferring energy from low (i.e., phyto and zooplankton) to higher (i.e., fish, seabirds, marine mammals, and other predators) trophic levels (Cury et al., 2000) (Figure 1). SPF are sensitive to environmental fluctuations due to its affinity for areas of high environmental variability (these zones of upwelling and areas of tidal mixing and river discharge (Teixeira et al., 2016)), which can be amplified by climate variability, implying cascading effects up and down the food web due to their high biomass at intermediate levels (mid-trophic-level position) and their potential wasp-waist control of the feeding network (Pennino et al., 2020b). In fact, fish abundance could go from several hundreds of thousands of tons to practically zero in the most extreme cases (Lluch-Belda et al., 1989). Besides the repercussions at the ecosystem level, the very strong fluctuations often have dramatic consequences for fishing communities, even across entire countries and regions (Allison et al., 2009; Teixeira et al., 2016).

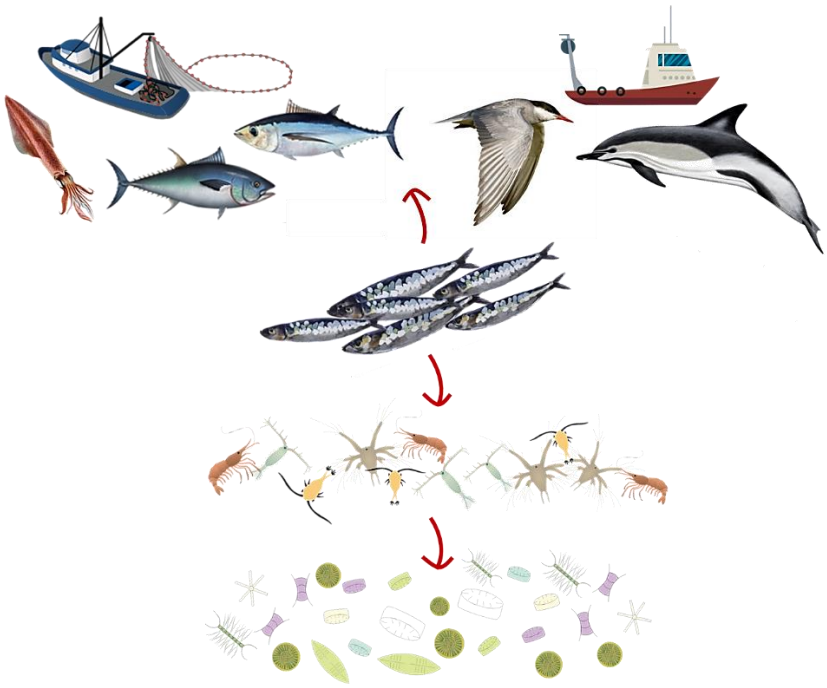


Figure 1. Wasp-waist control (up and down) exerted by small pelagic fish (forage species) in the food web of the ecosystem.

Among the SPF, sardines (considering genera *Sardina* and *Sardinops*) along with anchovies (*Engraulis*) are the most noteworthy species, since together made up 66.7 % of landings of small pelagic fish and 12 % of the major global captured species in 2022 (FAO, 2022), being usually found in species pairs in most systems (upwelling or non-upwelling) (Nikolioudakis et al., 2012).

Sardines are extensively fished in most temperate and productive world's coastal regions: off Japan and slightly off Australasia, and in the California, Humboldt, Benguela (mainly colonised by *Sardinops* genera) and Canary current systems (including the Mediterranean and the North Sea, represented by the genus *Sardina* with the species *Sardina pilchardus* (Walbaum, 1792), the protagonist of this thesis) (Van der Lingen et al., 2006; Checkley et al., 2017). *Sardinella* genus includes tropical and some subtropical sardine species (Parrish et al., 1989; Checkley et al., 2017), although in recent years the expansion of the distribution towards the north of one of the species of this genus, *Sardinella aurita* (Valenciennes, 1847), has been observed in the Mediterranean Sea and in Atlantic waters from Senegal to Mauretania and Morocco (Alheit et al., 2014; Bachiller et al., 2021).

Sardina pilchardus is widely distributed in the north-eastern Atlantic areas from the North Sea to Senegal, as well as in the Mediterranean Sea including its adjacent water bodies (Parrish et al., 1989), where accounts for around 15 - 20 % of the total marine captured production (Tsikliras & Koutrakis, 2013). Its role in the environment has been highlighted when it comes to their contribution in predators' diet, these being mainly barracudas *Sphyraena* spp., mackerels *Scomber colias*, greater amberjack *Seriola dumerili* and twaite shad *Alosa fallax* (Coll & Bellido, 2019).

2. Biological features of European sardine: reproductive and feeding ecology

The European sardine is a neritic small (< 25 cm) pelagic fish with temperate water affinity from the Clupeidae family. Like other small pelagics, they have an aggregative behaviour, rapid response to climate and environmental signals, and large variable natural mortality (Groenewald, 2021). They are, therefore, very dependent on the environment, and the way in which they store the energy has to be dynamic and easy to be allocated to cover the needs of maintaining structures, growth, as well as the processes of maturation and release of gametes (Figure 2).

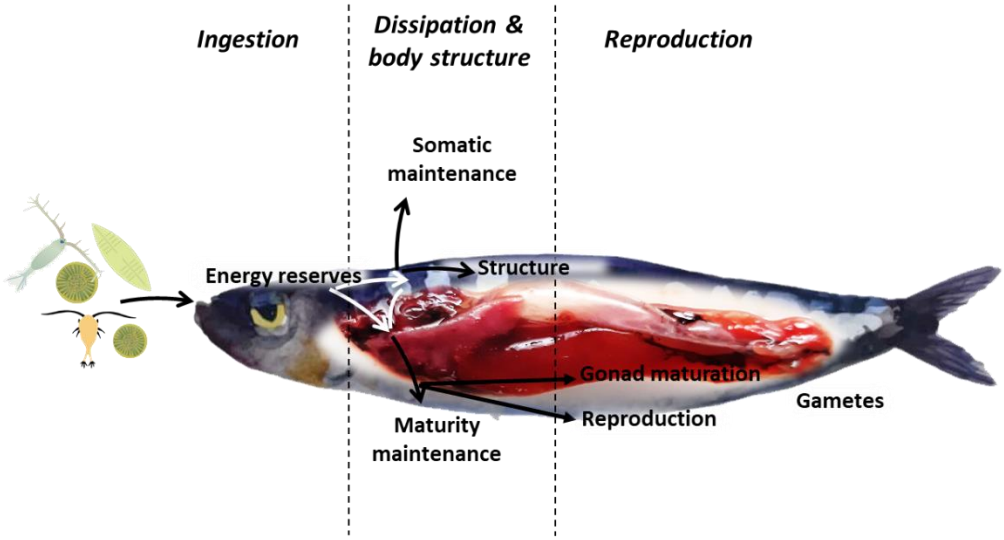


Figure 2. Diagram of the standard dynamic energy budget. A significant amount of energy is destined to the maturation of the gonads, reproduction and gametes production and spawn (adapted from Groenewald, 2021).

In that regard, sardine's reproduction is characterised by an early maturation, many eggs per body mass, and batch spawning (eggs are released more than once through a spawning season), common strategies to compensate for short life span (of about 5 years (Tsikliras & Koutrakis, 2013)) and lifetime fecundity (Nunes et al., 2011; Ganias et al., 2014), which starts during the end of the first year of life (Sinovčić et al., 2008). Furthermore, based on a well-established inverse relationship between the seasonal evolution of reproduction and the cycling of body condition and fat reserves, sardine follows a 'capital breeding' strategy (Ganias, 2009; McBride et al., 2015). The capital breeding system reduces the energetic conflict in fish species that participate in costly migrations to spawning grounds, or that stop feeding prior to or during spawning (McBride et al., 2015). It has been described as an energetic dependence of egg production on past resources, decoupling their spawning from prey bloom availability (Ganias et al., 2007; Groenewald, 2021), although in sardine the direct food input (main source in income breeders) seems to be significant for the production of gametes (Nunes et al., 2011). As species, the general pattern (Figure 3) is to store energy during spring-summer (regressing, regenerating, and developing gonadal stages) to be allocated later for the reproduction in autumn-winter (between October-November and March) (spawning capable and actively spawning stages), even though the reproductive phenology of sardine stocks may be closely linked to the area and its features. In fact, temperature seems to trigger gonad maturation (Tsikliras & Koutrakis, 2013), with

preferences for spawning at 14 –15 °C and avoidance for temperatures below 12 °C and above 16 °C (Stratoudakis et al., 2007). On the other hand, the amount of energy accumulated by the fish during the spring-summer period seems to be directly translated in more or less energy available for reproduction and, therefore, the spawning season may end when the available energy decreases below a certain threshold (Rosa et al., 2010), thus being variable at time and space.

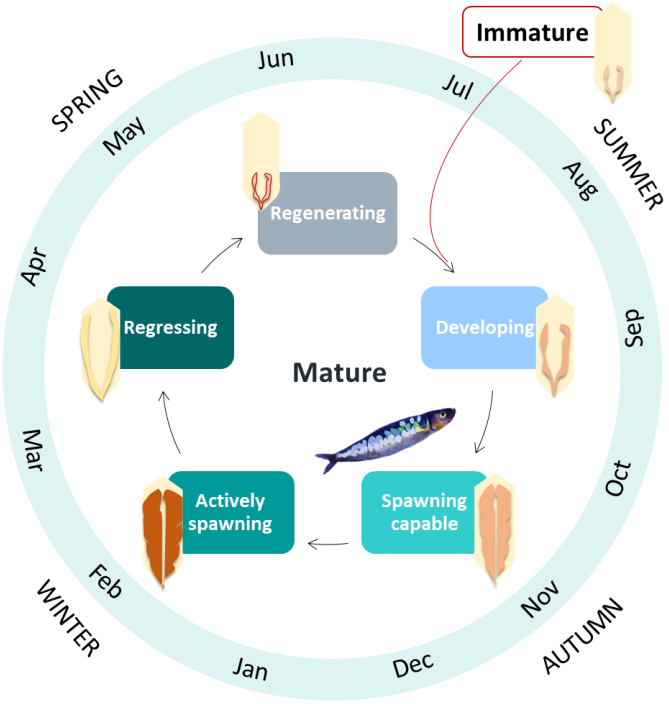


Figure 3. Gonadal developmental phases (following Brown-Peterson et al., 2011) according to the general reproductive cycle phenology of the European sardine.

Despite the variability according to the geographical distribution, life stage, and seasonality in terms of the zooplankton/phytoplankton proportion that sardines consume (Nikolioudakis et al., 2012; Costalago & Palomera, 2014), they mainly obtain the energy from zooplankton (especially smaller copepods and cladocerans), although protists and copepod nauplii and postnauplii are the main source during the larval stage (Morote et al., 2010). However, sardine has a highly plastic feeding behaviour as juveniles and adults, being able to use filter (non-selective) or particulate (selective) feeding over a broad prey size spectrum with high efficiency (Garrido et al., 2007a). The ability to switch between these feeding modes makes this species highly opportunistic, as it maximizes the energy intake by applying the most appropriate feeding strategy for a particular food environment (Costalago et al., 2015; Queiros et al., 2019).

The set of both reproductive and feeding characteristics closely dependent upon environmental conditions often make their populations excellent bio-indicators of climate-driven changes in marine systems world-wide (Peck et al., 2013), helping to develop a baseline that will allow to identify large-scale ecological patterns and tracking changes in ecosystem structure and function (Tsikliras & Koutrakis, 2013).

3. The study of sardine's body condition into the reproductive framework

The physiological health and energetic status of fish is increasingly evaluated for a broad range of purposes: from evaluating single species energy content and reproductive potential to developing multispecies ecosystem indicators (Wuenschel et al., 2019). In this way, the concept of 'condition' involves various aspects and can be interpreted according to the application that is to be granted. It is a term widely used to refer to the overall physiological and nutritional status or health of an individual and fish populations, and of central importance to many aspects of life history, including metabolism, reproductive potential, and mortality (Cone, 1989; McPherson et al., 2011; Schloesser & Fabrizio, 2017). Condition can be affected by both endogenous and exogenous factors, including the sex and reproductive status of the individual, the age, its latitude and depth, and further environmental variables and pressures (Lloret et al., 2002; Rosa et al., 2010).

Although other definitions are possible, condition indices are frequently considered to measure the extent of an individual's stored energy (McPherson et al., 2011). The form of storage diverges between taxonomic groups, so it is necessary to have notions about the energetic physiology of the species to study its condition. For example, an individual belonging to the clupeids (and more broadly to the pelagic species), as sardine, mainly stores energy in the muscles, while cod (Gadiformes, and more broadly the demersal species), mainly stores their energy in the liver (Brosset, 2016).

To quantify energy reservoirs, methodologies have been divided into two classes: morphophysiological (or 'indirect approaches') and physiological–biochemical (or 'direct approaches') (see Box 1). In the present thesis, indirect approaches have been used, as they are more practical and easier to apply, although direct approaches are usually more informative and accurate but they require more effort, are destructive, impractical for field sampling and large sample sizes, and more expensive (Crossin & Hinch, 2005; Wuenschel et al., 2019). In this way, we have combined morphophysiological approaches presented in

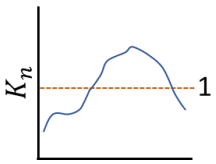
Box 1, in order to reach conclusions as close to the real condition of individuals without sacrificing the sample size of our studies.

Box 1. Resume of methods to measure fish condition and energy content.

Morphophysiological approaches

- Length-based indices

  Relative condition factor, Kn (Le Cren, 1951): the ratio of observed weight of a fish at a given length to the expected weight of a fish of the same length as calculated from the length weight regression. If Kn > 1, fish are considered to be in better condition as expected



- Organosomatic indices

  Energy invested in a certain function performed by an organ (organ weight) with respect to the weight of the individual - Hepatosomatic index, HSI, gonadosomatic index, GSI, etc.

$$\text{HSI} = \frac{Liver_W}{Weight} \quad \text{GSI} = \frac{Gonad_W}{Weight}$$

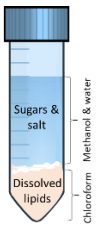
- Tissue properties

  Water content of tissues inversely relates to the amount of lipid stored in subdermal reserves (Schloesser & Fabrizio, 2017) - Distell fish fatmeter

Physiological–biochemical approaches

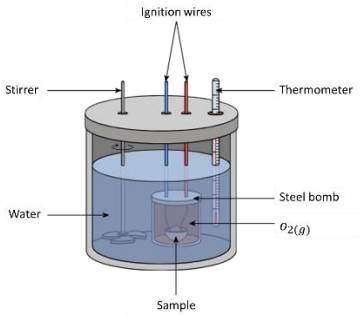
- Biochemical analysis – Folch method

  Based on the partitioning of lipids in a biphasic mixture of chloroform and methanol (Folch et al., 1957)



- Bomb calorimetry

  Directly estimates energy density by measuring the amount of heat released through combustion of dried tissue (Cummins & Wuycheck, 1971), made up of protein, lipid and carbohydrate



The relationship between condition and reproduction in European sardines is bidirectional. As discussed through the previous section, condition may reflect different aspects of an individual's physiology and may be affected differently by both the reproductive state and reproductive mode of a given species (Wuenschel et al., 2019), but also general condition and energy storage has a direct implication in its life history traits, involving reproduction, reflected through recruitment (Albo-Puigserver et al., 2017). In this regard, the capital breeding strategy (i.e., energy storage in spring-summer and spawning in autumn-winter) makes the condition of the individuals in a population highly variable throughout an annual cycle. Following the pattern of sardine, it would be expected that the energetic condition of the individuals would be higher in the months of reproductive rest and wide availability of resources (spring-summer), and lower when it is used directly in the physiological mechanisms involved in the maturation of gametes and their release (autumn-winter).

However, fish at different reproductive stages can coexist at the species level due to environmental reasons, their impact across generations, and individual variability (defined by the age, genetic characteristics, etc.). This coexistence can even occur at stock level, as maturation schedules in fish are considered flexible, dependent on the energy-allocation strategies, as well as on the nutrition or condition status (Lowerre-Barbieri, 2019). For this reason, and since the reproduction state define the condition (and the other way around), the evaluation of sardine health should be carried out taking into account the gonadal phase of the individuals. In this way, the temporary variable (month or season) will be complemented with the crucial variable in the state of condition, the reproductive stage, since the comparison between stocks taking into account the temporality could generate errors in the interpretation of the condition due, simply, to the different phenology in spawning. Also, an analysis of the condition of a single stock in a reproductive context would provide valuable information, allowing to study its internal variability and its sources of change. This approximation has already been purposed for other species, such as bluemouth rockfish (*Helicolenus dactylopterus dactylopterus*) (Muñoz & Casadevall, 2002) or Mediterranean ling (*Molva macrophthalma*) (Serrat & Muñoz, 2022).

4. Alarming trends of the European sardine in the Mediterranean Sea: emerging stressors

The Mediterranean Sea is a landlocked dynamic system with significant temporal and spatial variability, as it has limited exchange with the world ocean, an active deep overturning circulation, a shallow circulation cell and a convoluted upper layer circulation with several permanent and quasipermanent eddies (Tanhua et al., 2013). In the west, it connects through the Strait of Gibraltar to the Atlantic Ocean, and through the Dardanelles to the Sea of Marmara and the Black Sea in the northeast. In the southeast, the Suez Canal links the Mediterranean to the Red Sea and the Indian Ocean (Coll et al., 2010). Moreover, within the global ocean, the Mediterranean Sea is recognised as a biodiversity hotspot (Claudet et al., 2020). Unfortunately, the Mediterranean Sea has been considered one of the most impacted water masses in the world, as many disturbances interact synergistically with climate change (Halpern et al., 2008; Lejeusne et al., 2010; Chefaoui et al., 2018).

Fishing pressure in the Mediterranean together with the Black Sea presented the highest percentage (62.5 %) of stocks fished at unsustainable levels worldwide in 2017 among the FAO's 16 Major Fishing Areas (FAO, 2020). The last report of The State of World Fisheries and Aquaculture (FAO, 2022) shows that in 2019, the Mediterranean and Black Sea presented even a higher percentage of unsustainable fished stocks (63.4 %), although standing behind the Southeast Pacific (66.7 %). After reaching a historical maximum of about 2 million tonnes in the mid-1980s, total landings in the Mediterranean and Black Seas reduced to a low of 1.1 million tonnes in 2014; since 2015, they have recovered slightly, arriving to 1.4 million tonnes in 2019.

In fact, stocks of European sardine in the area (Figure 4), which are of great interest for many countries of the Mediterranean coast (Figure 5), continue to be fished outside biologically sustainable limits, as occurs with most of the commercially important stocks regularly assessed (e.g., hake (*Merluccius merluccius*), turbot (*Scophthalmus maximus*)) (FAO, 2022).

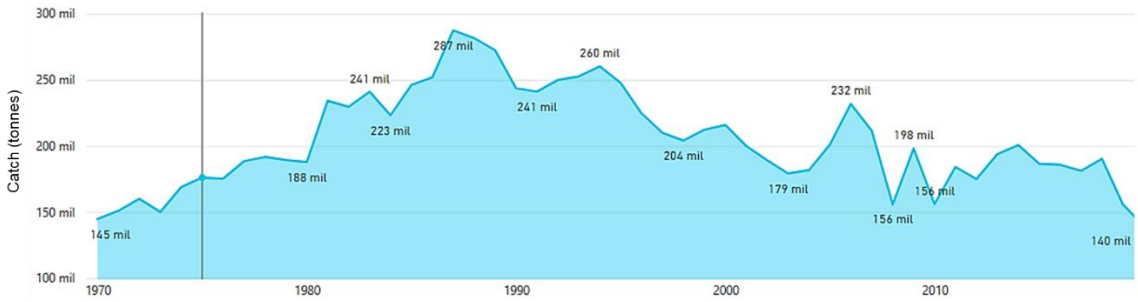


Figure 4. Trend of European sardine's capture production (in tonnes) from 1970 to 2020 in the Mediterranean and Black Sea basins. Source: FAO/GFCM (2022) based on data collected by national authorities through the STATLANT 37A questionnaire https://www.fao.org/gfcm/data/capture-production

Following the general trend of captures in the Mediterranean and Black Seas, the maximum production (287,329 tonnes in 1987) was reached between the years 1980 and 1990, suffering a large decline afterwards throughout the 1990s (Figure 4). Despite sporadic peaks in subsequent years, sardine biomass caught has followed a downward tendency in recent decades.

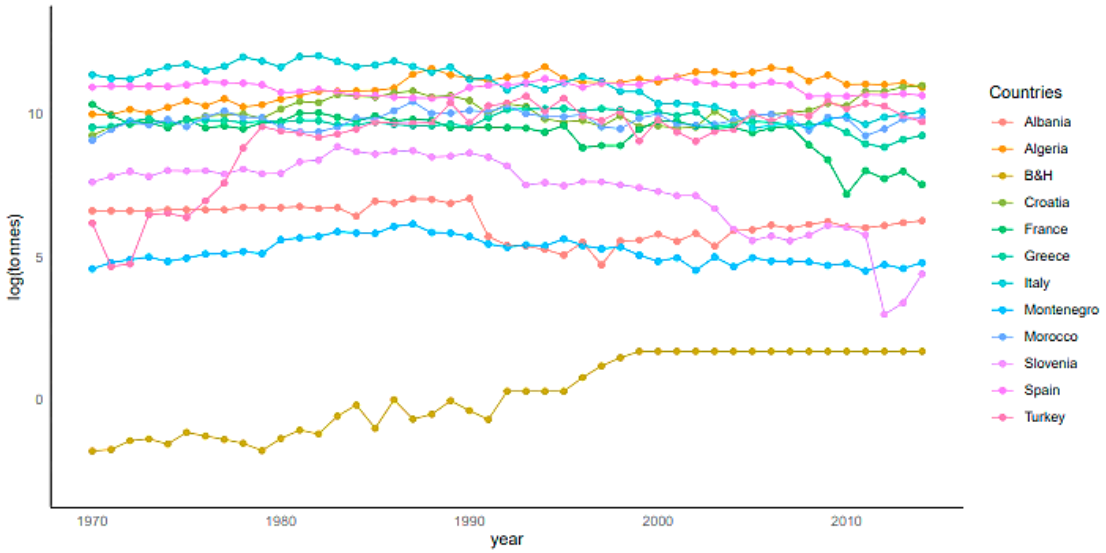


Figure 5. Total annual log tons of European sardine (*Sardina pilchardus*) caught per country, from 1970 to 2014. Source: Calvo et al. (2020).

Furthermore, despite the differences along the basin with a north-eastern area with a higher thermal anomaly (Figure 6A), the Mediterranean ranks among the fastest warming ocean regions due to its semi-enclosed nature (Marbà et al., 2015; Chefaoui et al., 2018), rising the periodicity and severity of extreme events (Di Biagio et al., 2020) (e.g., marine heat waves during summer 2022, reaching over 30 ºC in some points).

The increasing temperature (Figure 6B) does not only have a direct action on the physiology and behaviour of individuals, but also on water stratification (Calvo et al., 2011), plankton productivity and, definitely, on fish resources availability (Brosset et al., 2017), which should be concerned even more as it is considered an oligotrophic sea, although regional features enrich coastal areas through changing wind conditions, temporal thermoclines, currents and river discharges, and municipal sewage (Coll et al., 2010). Limited resources are connected with the potential competition for food with other species with higher thermal affinity and feeding plasticity that occupy similar trophic niches, such as round sardinella (*Sardinella aurita*), which has increased considerably at the same time that the European sardine suffers from instability and a general downward trend (Bachiller et al., 2021).

On the other hand, temperature have important effects on parasitism and disease (Marcogliese, 2008). Climate warming potentially affect host–pathogen interactions by (i) increasing pathogen development rates, transmission and number of generation times per year, (ii) relaxing overwintering restrictions on pathogen life cycles, and (iii) making more pronounced the host susceptibility to thermal stressors (Harvell et al., 2022). In fact, warming of coastal waters may result in a higher number of pelagic fish species that follow warmer currents northwards, increasing fish infection by the ascaridoid nematode *Anisakis* spp. (Klimpel & Palm, 2011). In addition to the negative perception and potential risks caused by parasites such as nematodes in the fish product, they can negatively act on the health of the fish, affecting host physiology, morphology, reproduction and behaviour (Timi & Poulin, 2020), such as severe inflammatory reactions with tissue deformation in the Atlantic cod or haemorrhages and inflammation around the vent in salmonids, as attributed to the ascaridoid nematode *Anisakis simplex* (Buchmann & Mehrdana, 2016). In this sense, there is a lack of information on the current parasite load of European sardines by nematodes and their link with host condition status, which makes it difficult to monitor over time and study the potential effect of climate change after this issue.

Thus, for all the reasons mentioned, temperature is a dominant environmental driver for predicting pelagic fishery health (Lloret et al., 2021).

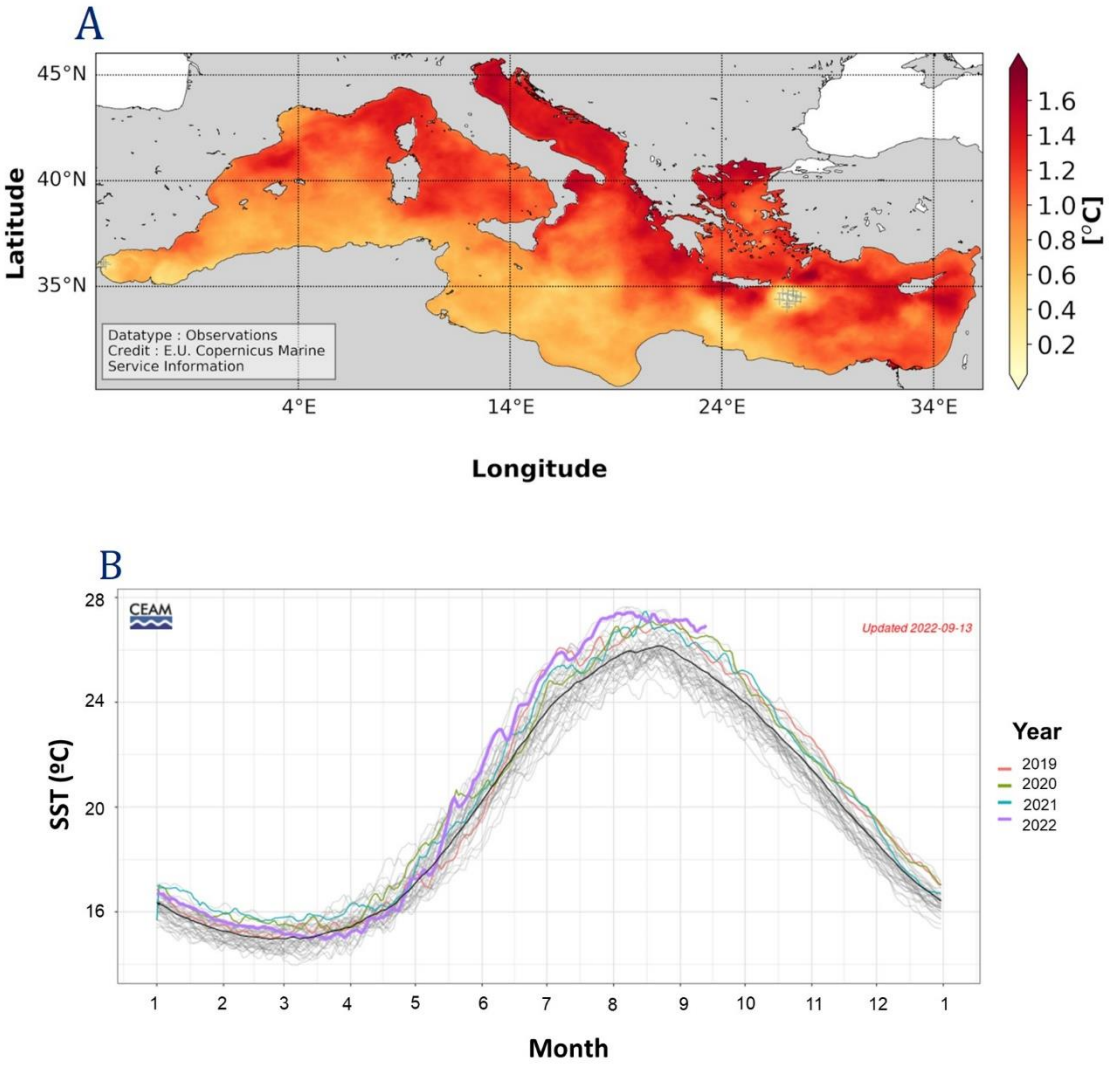


Figure 6. A, B. Mediterranean warming over the last decades. **A.** Sea surface temperature cumulative trend over the period 1993-2020 in the Mediterranean Sea. The cumulative trend is the rate of change (°C/year) scaled by the number of time steps (28 years). Source: E.U. Copernicus Marine Service Information (2022); **B.** Mediterranean daily SST (ºC) from 1982 to 2022. Average is represented by a black line and from 2019 on, years are highlighted with colours. Source: CEAM (2022) http://www.ceam.es/ceamet/SST/SST-trend.html.

Other threaten that should be mentioned is pollution in all its forms (i.e., marine litter and microplastics (García-Rivera et al., 2018; Sala et al., 2022), marine noise pollution (Di Franco et al., 2020), etc.), as the Mediterranean Sea is one of the most affected areas in the world, probably related to the high level of anthropogenic activities in surrounding areas (Pennino et al., 2020a), a semi-closed basin shape and the oceanographic regimes.

In this context, there are many historical and emerging factors that affect small pelagic fish and, specifically, the health of Mediterranean sardine stocks, as reported in several

studies. In fact, in the last decades small pelagic fish stocks in the Mediterranean have been suffering from population declines and some of them have shown signs of collapse (Coll & Bellido, 2019; Ramírez et al., 2021). Sardine's decrease in biomass and catches has been reported in different subregions of the Mediterranean Sea, linked to a decline in body length, a slower growth, the disappearance of older individuals, and worse body condition (e.g., Sinovčić et al., 2008; Van Beveren et al., 2014; Quattrocchi & Maynou, 2017; Brosset et al., 2017; Şenbahar et al., 2020) (Figure 7).

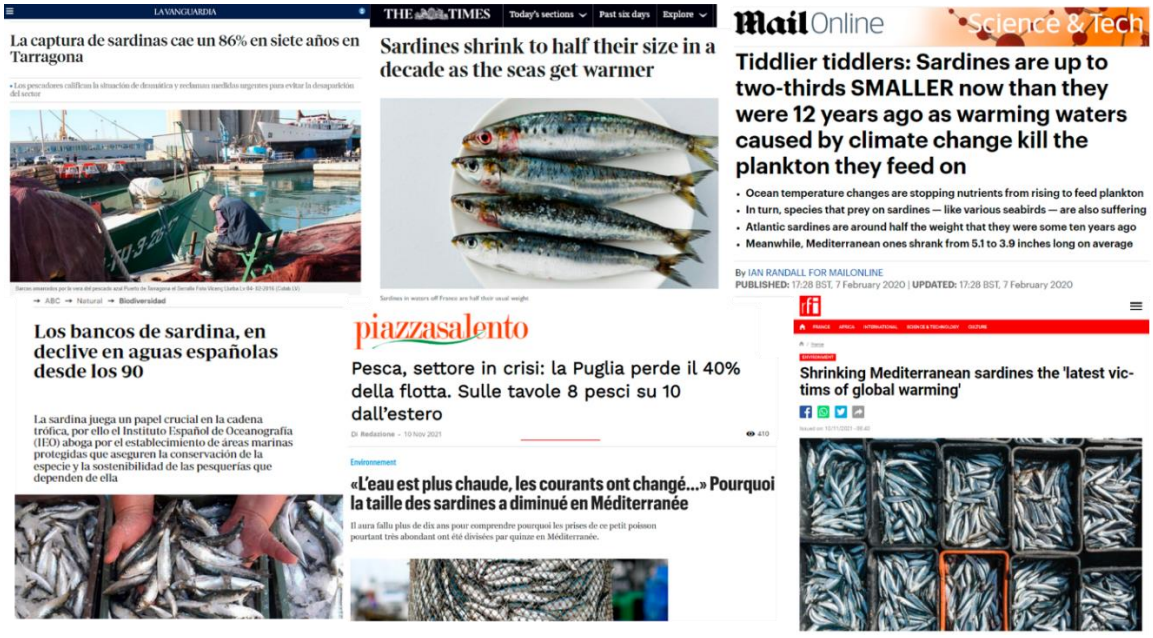


Figure 7. News of the poor state and catches decline of the European sardine as a fishing resource in the Mediterranean. Source: ABC, 2012; La Vanguardia, 2016; The Times, 2020; MailOnline, 2020; Piazzasalento, 2021; Le Parisiene, 2021; rFI, 2021.

5. The importance of delimiting genetic units in the assessment of small pelagic fish condition

The contribution to an efficient management of fishery resources is only possible within a context of biologically and ecologically relevant processes that allow to study each stock taking into account its particularities. In this sense, a prerequisite in fisheries science is to identify biological meaningful delineations of stocks (Reiss et al., 2009; Huret et al., 2020) with the aim of providing information to management that ensures the sustainability of the fishing resource by making them match the fundamental exploited units. Thus, the evaluation of the condition and ultimately, the health status, as well as of the reproductive phenology, investment, and potential, should be carried out taking into account the

biological units. If predetermined barriers (that frequently were established taking into account economic, social, and/or political criteria) based on management strategies that do not totally overlap population units are applied, the study of the status of each population would be masked, generating inaccurate conclusions. In fact, severe problems could be aroused when populations are aggregated and evaluated at the scale of the management unit, causing its decline to even reach extinction (Frank & Brickman, 2000).

However, delimiting biological units is not an easy task (Cadrin, 2020), especially in small pelagic fish, as geographical boundaries in the marine environment are diffuse for species with high dispersal potential at every life stage (larvae dispersion, and juveniles and adults following seasonal migration patterns for spawning and feeding) and large population sizes. Actually, it was traditionally thought that pelagic marine fishes exhibit genetic homogeneity, and even random mating (Moore & Chaplin, 2013). Nevertheless, in recent decades, and mainly due to the incorporation of genetics in fisheries, this statement has been denied after the demonstration of the population subdivision in a large part of the species.

To identify natural environmental influences on the evolution of the phenotype is important to anticipate how populations might adaptively respond to human activities such as fishing, global environmental change, habitat changes and conservation actions (Naish & Hard, 2008). Nevertheless, in addition to the environmental variability expected throughout the Mediterranean (which may also contribute to shaping genetic units through selection processes (Kasapidis et al., 2012)), the genetic distance between sardine populations seems to be decisive in the existence of differences in parameters such as growth, morphometric characters, and length of first maturity throughout sardine's Mediterranean distribution (Silva et al., 2008; Dimarchopoulou & Tsikliras, 2022), considered as phenotypic plasticity. Notwithstanding, there is no clear consensus on the genetic stock structure of *S. pilchardus*, what complicates the task of stock assessment with a biological/populational meaning. Thus, one of the challenges of the present doctoral thesis was to integrate sardine's genetic information available to date to define the most plausible biological/genetical units (as intended by **Chapter 1**, published as Caballero-Huertas et al. (2022a)), to subsequently carry out coherent studies of the current state of the sardine along the Mediterranean (developed in the following chapters).

Objectives and structure of the thesis

During the last decades, there has been an increasing interest to the scientific and management communities to find the principal sources of pressure that have been causing the fall of biomass and condition in European sardine stocks in the Mediterranean. Anthropogenic pressure, which implies fishing exploitation together with further environmental pressures, involving the increase in sea temperature caused by climate change and its derived effects, have been singled out for having a leading role in this regard.

However, there are still many unanswered questions in this aspect, in part due to the lack of integration of genetic information and the delimitation of sardine populations, and of the environmental factors that condition the health status of this resource along its distributional range. This is added to the few studies that address the somatic condition of sardine into a reproductive framework, as well as that consider and compare the current variability in the health of different stocks due to multiple synergistic actors and features.

Some of these questions are the following:

- How are the European sardine stocks genetically structured and what is known about the local adaptation processes?
- What is the current condition and lipid storage status in the Mediterranean sardine?
- What is the state of the condition in the transition zone with the Atlantic?
- How is the current condition status related to the reproductive cycle?
- What are the main factors influencing condition and reproduction in the Mediterranean?
- What is the current parasitic load of the sardine and how does this influence the condition?

In order to contribute to the resolution of these questions, among others, this thesis aims to shed light on the current health status of the European sardine from a holistic point of view, approaching the concepts mentioned above (i.e., genetic variability and selection mechanisms, somatic condition, reproduction, and parasitism) and connecting them in an environmental setting. Moreover, this work compiles chapters in sardine health status at local level, but also studies based on the comparison among stocks, taking into account diverse environmental factors that influence the different study areas (especially, Mediterranean sampling locations, but also a nearby Atlantic location as external point). The approach presented here has been designed with the interest of pointing out all the aspects to be considered so that the management of this important fishing resource is as efficient as possible. In this way, it is expected that the purely scientific information is at the

service of the management for the conservation of a species of great ecological and fishing relevance.

Therefore, the **general objective** of this doctoral thesis is to determine the current spatial and seasonal variability of the health status of the European sardine within the reproductive context considering biological/genetic stocks (genetic variability) and evaluating the effect of the environmental factors and their heterogeneity throughout the Mediterranean Sea. Additionally, the implementation of the results obtained in sardine fishery management is proposed.

To achieve the general objective, the following specific objectives have aroused:

1.- To establish the most plausible E. sardine genetic stocks/units according to literature.

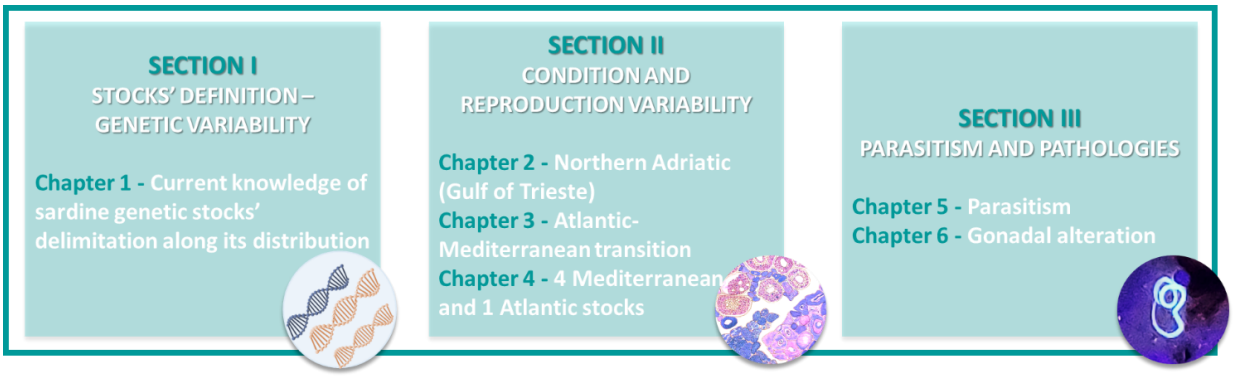
2.- To analyse the current health status (mainly by the assessment of condition) of different sardine stocks specimens throughout the seasons and reproductive cycle, with special emphasis in the Mediterranean and the Atlantic-Mediterranean transition.

3.- To compare condition parameters among E. sardine stocks from a genetic and environmental perspective.

4.- To identify the abiotic and biotic causes behind the decline in sardine condition.

5.- To suggest the application of improvements in the management of the sardine fishery.

To address the mentioned aims, six chapters have been developed, which are collected in three different sections, although all of them are essential to approach conclusions that respond to our starting objectives. In order to carry out an appropriate analysis of the health status of sardine along a great part of its range of distribution, it has been necessary to determine firstly the biological/genetic units that make up the current different stocks or potential populations (Section I - **Chapter 1**). Secondly (Section II), exhaustive analyses of the somatic condition linked to the reproductive traits (especially, phenology) have been carried out in individual stocks (Section II - **Chapter 2**), by comparing stocks of the Atlantic-Mediterranean transition area (Section II - **Chapter 3**), as well as through a general study that includes stocks from various localities (Section II - **Chapter 4**) along E. sardine's Mediterranean distributional range. These three chapters have been developed considering the environmental variability and context in which the E. sardine is found. Finally, parasitism and potential reproductive pathologies have been analysed as possible factors that could be related with the state of condition or affecting sardine's state of health (Section III – **Chapter 5** and **6**).



Box 1. Diagram of thesis structure.

Thus, **Chapter 1** of this thesis explores the current potential genetic structure of the E. sardine by making use of the available published data and discussing the different hypotheses raised so far along its distributional range and the selection processes shaping genetic units. Also, we compare methodologies and genetic markers used to date in this regard. Moreover, we also integrate the genetic information available within the context of the present-day sardine fisheries management. This chapter was published in a peer-reviewed scientific journal as **Caballero-Huertas, M.**, Frigola-Tepe, X., Coll, M., Muñoz, M., & Viñas, J. (2022). The current knowledge status of the genetic population structure of the European sardine (*Sardina pilchardus*): uncertainties to be solved for an appropriate fishery management. *Reviews in Fish Biology and Fisheries*, 1-19. DOI: 10.1007/s11160-022-09704-z.

In relation to the somatic condition analyses into a reproductive context, **Chapter 2** examines the role of different lipid reserves in European sardine as a capital breeder in the northernmost section of the most septentrional semi-enclosed basin of the Mediterranean (North Adriatic Sea, specifically, in the Gulf of Trieste, relevant in the Italian capture of sardines). Moreover, the effect of some environmental variables on the physiological and reproductive status of this fish stock is studied. We can find this chapter as an article in a special issue of the peer-reviewed journal *Fishes* as **Caballero-Huertas, M.**, Frigola-Tepe, X., Viñas, J., & Muñoz, M. (2022). Somatic condition and reproductive potential as a tandem in European sardine: an analysis with environmental perspective in the Northern Adriatic (Gulf of Trieste). *Fishes*, *7*(3), 105. DOI: 10.3390/fishes7030105. **Chapter 3** has as objective to seasonally characterise the differences in the energetic body condition as well as in the reproductive cycle between European sardines from two nearby and connected areas, the Southern Portugal-Gulf of Cádiz (Atlantic Ocean) and the Alboran Sea (Mediterranean Sea) coasts, and to identify the environmental variables that may influence the above parameters in both sardine stocks. Specifically, this study has been published in a peer-reviewed journal

with the reference **Caballero-Huertas, M.**, Vargas-Yánez, M., Frigola-Tepe, X., Viñas, J., & Muñoz, M. (2022). Unravelling the drivers of variability in body condition and reproduction of the European sardine along the Atlantic-Mediterranean transition. *Marine Environmental Research*, *179*, 105697. DOI: 10.1016/j.marenvres.2022.105697.

Considering the Mediterranean environmental variability from the west to the east, the comparison of sardine's condition indices within the reproductive framework is carried out in **Chapter 4**. This chapter highlights notable findings, such as the modulation of reproductive strategy or the role of the liver in sardines. It was submitted to the peer-reviewed journal *Frontiers in Marine Science* under the reference **Caballero-Huertas, M.**, Frigola-Tepe, X., Viñas, J., & Muñoz, M. From west to east: heterogeneity in the life history traits of a small pelagic fish (*Sardina pilchardus*) throughout the Mediterranean.

In the section of parasitism and pathologies, **Chapter 5** defines the nematode (order Ascaridida) load and the features of the infection along sardine's Mediterranean distribution, and relates condition parameters and reproductive features with these parasites. Different methodologies are combined in this chapter, as UV-press and direct sequencing of nematode DNA with approaches to determine sardine's health status. Its content could be read in a peer-reviewed journal, reference **Caballero-Huertas, M.**, Palomba, M., Frigola-Tepe, X., Muñoz, M., Mattiucci, S., & Viñas, J. (2023). Ascaridoid parasites in European sardine throughout the annual cycle: Variability in parasitic load according to host stock features. *International Journal for Parasitology: Parasites and Wildlife*, *20*, 1-11. DOI: 10.1016/j.ijppaw.2022.12.001. And, finally, in **Chapter 6** we describe for the first time a testicular anomaly, the gonadal segmentation, in sardines from three Mediterranean areas (Northern Spain, Northern Adriatic Sea, and Aegean Sea). Its cause is still unknown, even though parasitism itself or chemical exposure may be under this event. This research is found published in a peer-reviewed journal as **Caballero-Huertas, M.**, Frigola-Tepe, X., Viñas, J., & Muñoz, M. (2022). Evidence of trilobed testes in European sardine (*Sardina pilchardus*). *Cybium*, *46*(1). DOI: 10.26028/cybium/2022-461-004.

Methodology

This part of the thesis outlines the materials and methods used for its development. The basic information on the study area and main methodological procedures are described in a generalised way. Some of the procedures, parameters and indices here described apply for almost all chapters, while others are specific of one of them. Thus, further details on the specific methodology as well as the data analysis are provided in each chapter of the Results section.

1. Samples' collection

To obtain a representativeness of the European sardine during its annual cycle throughout the Mediterranean, total of 3101 specimens of *Sardina pilchardus* were collected seasonally (Table 1), being seasons defined as winter: January, February, March; spring: April, May, June; summer: July, August, September; autumn: October, November, December, from 2019 to 2021 throughout four Mediterranean sub-areas (GSA), following the General Fisheries Commission for the Mediterranean (GFCM) delimitations established for stock assessments by commercial fisheries: GSA 1-Northern Alboran Sea, and GSA 6-Northern Spain (Western Mediterranean Sea); GSA 17-Northern Adriatic (Central Mediterranean Sea); and GSA 22-Aegean (Eastern Mediterranean Sea). An external point to the Mediterranean Sea was selected, located in the Northeast Atlantic Ocean according to the subareas and divisions of FAO fishing areas (Portuguese Waters - East (FAO fishing area division 27.9.a)) (Figure 1).

The fish came from purse seine fisheries for commercial purposes. Immediately after the purchase, samples were frozen at - 20 ºC, which has been demonstrated that it does not affect condition variables (Brosset et al., 2015a). Excepting 32 individuals of the total (1.03 %), all specimens in this study respected the minimum landing size for sardine (total length (L_T) of ≥11 cm)) in the Mediterranean Sea (Popescu, 2018).

Table 1. Summary of all the samples analysed in the thesis. Atl: Atlantic Ocean; Med: Mediterranean Sea; N: number of individuals; SD: standard deviation within the group. Parentheses indicate the minimum and maximum value per group.

Area	Season	N	Mean total length ± SD (cm)
South Portugal (FAO Division 27.9.a) Atl	Winter (Feb, Mar)	189	18.372 ± 0.918 (13.5 - 20.4)
	Spring (May, Jun)	204	16.859 ± 1.314 (13.9 - 20.1)
	Summer (Sep)	101	17.502 ± 1.185 (14.4 - 21.0)
	Autumn (Oct)	105	18.486 ± 0.815 (16.6 - 22.0)

Northern Alboran (GSA 1) Med	Winter (Feb)	20	19.235 ± 0.908 (15.9 - 20.4)
	Spring (Apr, Jun)	208	14.958 ± 3.547 (9.1 - 20.4)
	Summer (Aug)	99	16.303 ± 0.941 (10.8 - 19.1)
	Autumn (Oct, Nov)	202	16.522 ± 3.149 (11.1 - 23.0)
Northern Spain (GSA 6) Med	Winter (Feb, Mar)	233	13.427 ± 1.083 (10.8 - 17.3)
	Spring (May, Jun)	110	13.144 ± 1.012 (9.5 - 16.0)
	Summer (Jul, Aug)	105	13.635 ± 1.016 (10.9 - 15.4)
	Autumn (Oct, Nov, Dec)	204	14.627 ± 1.068 (11.7 - 17.6)
Northern Adriatic Sea (GSA 17) Med	Winter (Jan, Feb)	202	13.811 ± 0.944 (11.2 - 16.0)
	Spring (May, Jun)	200	12.775 ± 0.586 (11.0 - 14.5)
	Summer (Sep)	101	13.391 ± 0.613 (12.3 - 15.6)
	Autumn (Nov, Dec)	201	13.497 ± 0.832 (10.7 - 15.6)
Aegean Sea (GSA 22) Med	Winter (Jan, Feb)	216	12.134 ± 0.865 (10.6 - 16.3)
	Spring (Jun)	102	12.797 ± 0.530 (11.7 - 14.5)
	Summer (Jul)	100	13.794 ± 0.577 (12.3 - 15.6)
	Autumn (Oct, Nov, Dec)	199	13.205 ± 1.078 (10.2 - 15.5)

2. Description of the study area

In order to carry out a representative study of the species in terms of genetic variability and population determination, we have covered all the available information throughout its distributional range, previously described. To study its current health status (condition, reproduction, and nematode parasitism) with the main focus on the Mediterranean, we have analysed individuals belonging to five locations (Figure 1). Sardines belonged to the FAO major fishing area 37, Mediterranean and Black Sea, following Geographical Sub-Areas (GSAs) established by the General Fisheries Commission for the Mediterranean (GFCM, 2009) according to the Resolution GFCM/33/2009/2: GSA 1-Alboran, GSA 6-Northern Spain (Western Mediterranean Sea); GSA 17-Northern Adriatic (Central Mediterranean Sea); and GSA 22-Aegean (Eastern Mediterranean Sea). On the other hand, we have analysed sardines from an external point of the Mediterranean, the FAO major fishing area 27, specifically, the South of the Portuguese Coast Subarea 27.9.a (Atlantic Ocean).

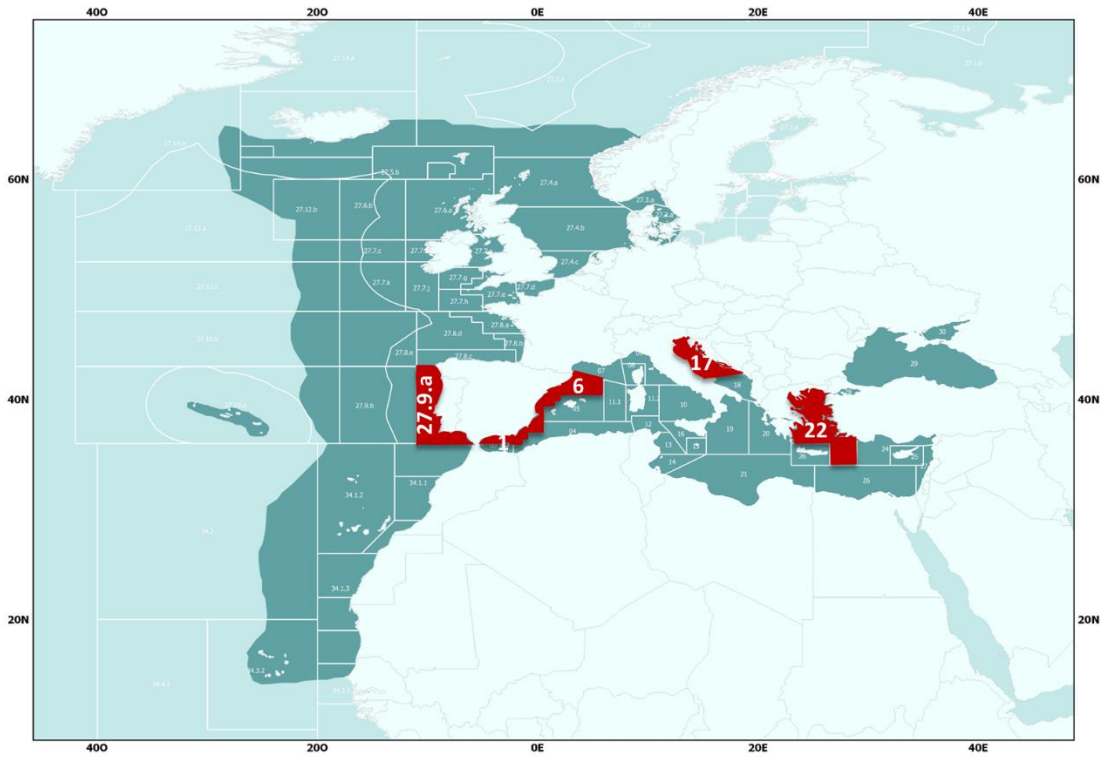


Figure 1. Distributional range of E. sardine (*S. pilchardus*) (darker blue) and the subareas from which individuals were extracted for the analysis of condition, reproduction, parasitism, and further aspects (red colour).

The Northern Alboran Sea subarea (GSA 1) is located in the southeast of the Iberian Peninsula, in the western Mediterranean Sea. It constitutes a transitional zone between Mediterranean and Atlantic waters, which are connected by the Strait of Gibraltar. In this area, there is an intense influence of the circulation, with a strong frontal area that divides a productive sector of intense upwelling in the coastal zone and a more oligotrophic area far offshore (Vargas-Yáñez et al., 2017, 2019; García-Martínez et al., 2019). Its hydrology is defined primarily by two main events: the Atlantic Jet, an input of surface waters through the Gibraltar strait which generates anticyclonic patterns in the current system, and the Almería–Orán front, which forms at the meeting point of Atlantic and Mediterranean surface waters, at the eastern end of the Alboran Sea (Abad et al., 2007). *Sardina pilchardus* represents the main pelagic species of the purse-seine catch in this area (Ramírez et al., 2001).

The Northern Spain area (GSA 6) is in the North-western (NW) Mediterranean, and it comprises the Catalan Sea and the Gulf of Valencia. The NW Mediterranean is a rather productive area due to the upwelling activity influenced by wind conditions and the fresh water inputs from the Rhone and Ebro Rivers (Agostini & Bakun, 2002; Corrales et al.,

2015). The commercial exploitation of small pelagics in the area has been significant since the early 1940s, with catches dominated by sardine, probably due to its coastal distribution, and constituting one of the most productive Mediterranean sardine stocks (Palomera et al., 2007; Silva et al., 2008).

The Northern Adriatic Sea (GSA 17) is in the northernmost section of the most septentrional Mediterranean sub-basin. It is one of the major chlorophyll hot spots in the Mediterranean Sea, as it is influenced by the nutrient discharge from the Po River and a dozen small rivers that flow into the Adriatic Sea north of the Po River delta and in the Gulf of Trieste, with around 40 % of the chlorophyll production of the whole Adriatic (Rizzi et al., 2016). Small pelagic fishing by trawlers and purse seiners (mainly focused on anchovy and sardine) is mostly concentrated in the northern part of the Adriatic Sea (Carpi et al., 2017).

The North Aegean Sea (GSA 22), the easternmost subarea included in this study, connects with the Black Sea through the Bosporus Straits, the Marmara Sea and the Strait of Dardanelles. It has the widest continental shelf and is the most productive region of the Aegean (Somarakis et al., 2002), as it is influenced by Black Sea waters and large rivers, constituting one of the most important small pelagic fishing grounds in the eastern Mediterranean (Somarakis & Nikolioudakis, 2007).

European sardines from the external sampling point (27.9.a) were mainly collected along the Southern Portugal and Gulf of Cádiz. This area is characterised by a notable productivity mainly due to rivers discharges, since the effects of upwelling are reduced and occasionally occur under the influence of local westerly winds, or when upwelled waters from the west Portuguese coast intrude over the southern shelf break following an easterly extension of the equatorward upwelling current (Garrido et al., 2008a). Sardine is the main target species of the purse seine fleet, contributing around 98 % of the landings in this division (Leitão et al., 2014).

3. Main analytical methods

3.1. Literature review (Chapter 1)

An extensive literature research of the state of the art of E. sardine's population structure was carried out. It went beyond mere description about this issue, intending to analyse, hypothesize and discuss all relevant aspects regarding the genetic structure and

selection processes shaping sardine's genetic units using all the available bibliography mainly found through the platforms Google Scholar, PubMed® and ResearchGate.

3.2. Morphophysiological approaches to evaluate sardine's condition (Chapters 2, 3, 4, 5 and 6)

Morphogravimetric measurements, length-based indices, organosomatic indices and measurements based on tissue properties were conducted in European sardine samples to estimate the energetic fitness (condition).

Length-based index

Le Cren's relative condition factor (Kn) (Le Cren, 1951) measures the deviation of an individual from the averaged weight at length in the respective sample, as follows:

$$\mathrm{Kn} = \frac{W_E}{W_r} = \frac{W_E}{\alpha {L_T}^{\beta}}$$

where W_E is the eviscerated body weight of an specimen, and W_r is the predicted eviscerated weight of an individual of a given total length. L_T is the total length, and α and β are coefficients obtained by the regression line of the logarithms of length and mass. It is considered as higher-than-average when Kn exceeds 1 for a given individual, and lower when it is under this value.

Organosomatic indices

As a measure of energy storage, hepatosomatic index (HSI) was calculated from the liver weight (W_L) as HSI = $100 \cdot W_L W_E^{-1}$.

Energy allocated to reproduction or the reproductive effort (Brosset et al., 2016) was estimated through the gonadosomatic index (GSI) from the gonad weight (W_G) as GSI = $100 \cdot W_G W_E^{-1}$.

Measurements based on tissue properties: tissue fat content

Total tissue fat content (i.e., total lipids in the muscle) was estimated by the average of both sides along the fish lateral line using a fish fat meter (Distell Model FM 992) (Kent, 1990) calibrated for European sardine (with SARDINE-2). It has been designed to measure the water content and provide the relative tissue fat content (%) of each individual due to the inverse relationship between water and lipid content (Bayse et al., 2018; Brosset et al., 2015a).

Besides, a visual scale for fat mesenteric reserves (Van Der Lingen & Hutchings, 2005) was used, where 1= fat lines invisible or thin and indistinct; 2 = depth greater than width of one or more fat lines; 3 = pyloric fat line noticeably thicker than the other fat lines, and about one-third the thickness of the pyloric junction; 4 = depth greater than width for all fat lines but no fat lobes present; 5 = all fat lines slightly lobed, but no overlap between lobes; 6 = fat line lobes obvious and show some overlap; and 7 = fat line lobes large, lots of overlap, and fundulus well-covered with fat.

3.3. Reproductive analysis (Chapters 2, 3, 4, 5, and 6)

Gonad developmental stages

Sex and gonadal development phases were macroscopically determined using standardised terminology described in Brown-Peterson et al. (2011) as immature (sardine has not reached sexual maturity), regenerating (mature but reproductively inactive), developing (gonads increasing in size with gametes that are beginning to develop), spawning capable (ready for the reproduction but sardine has not begun to spawn), actively spawning (sardine is releasing eggs), and regressing (gonads almost empty of gametes).

GSI was also applied, in this case, for supporting macroscopical determination of gonadal stages during the progress of the reproductive cycle.

Gonadal histology (Specific to Chapter 6)

Gonads were removed and fixed in 4 % formaldehyde buffered with $Na_2HPO_4 \cdot 2H_2O$ (0.046 M) and $NaH_2PO_4 \cdot H_2O$ (0.029 M). A subsample from the central section of the three lobes of the gonad of each individual was extracted, dehydrated and embedded in paraffin to be sectioned at 3 μm and stained with haematoxylin–eosin for its examination under microscope.

3.4. Ascarioid examination, infection parameters and species identification (Chapter 5)

UV-press method

Firstly, the visual inspection of sardine gills and the opening of the stomach and intestine to expose their content were carried out, using forceps and a lamp by naked eye. Then, the whole digestive tract including the pyloric caeca, liver and gonads were placed into a transparent 1-3 mm plastic bag next to the flesh cut into butterfly fillets. Bags were

then pressed under hydraulic pump and stored overnight at −20 °C for further inspection by UV-press method, which is based on the fluorescence of frozen ascaridoid larvae, allowing the visual inspection of flattened/pressed and subsequently deep-frozen fish fillets or viscera under UV-light exposure at 366 nm in a darkened room (Cipriani et al., 2018; Levsen et al., 2018; Mattiucci et al., 2018). Afterwards, all parasites were counted, and their anatomical location was reported.

Infection parameters

Quantitative Parasitology 3.0 software (Reiczigel et al., 2019) was applied to calculate the infection levels of *Anisakis* spp. and *Hysterothylacium* spp. larvae by sardine sampling area. General prevalence (P, %) with confidence limits (Clopper–Pearson interval, confidence level of 95 %), mean intensity (mI) (Bootstrap BCa with confidence level of 95 %, 2000 replications), and mean abundance (mA) (Bootstrap BCa with confidence level of 95 %, 2000 replications) were obtained.

Ascaridoid species identification by direct sequencing

A visual identification of the ascaridoid nematode to genus level based on morphology was carried out following Moravec (1994) and Berland (1961) criteria. Subsequently, total DNA from each specimen was extracted using a Quick-gDNA MiniPrep (Zymo Research Corp, CA, USA), following the manufacturer's protocol. The ITS region of the rDNA including the first internal transcribed spacer (ITS-1), the 5.8S gene, the second transcribed spacer (ITS-2), and ~70 nucleotides of the 28S gene, was amplified using the primers NC5 (forward, 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse, 5′-TTAGTTTCTTTTCCTCCGCT-3′) as reported in Zhu et al. (2000). PCRs were carried out following the protocol reported in Palomba et al. (2021). Additionally, the mitochondrial cytochrome c oxidase subunit II (mtDNA cox2) gene locus was amplified using the primers 211F (forward, 5′-TTTTCTAGTTATATAGATTGRTTYAT-3′) and 210R (reverse, 5′-CACCAACTCTTAAAATTATC-3′). PCRs were carried out following the protocol reported in Mattiucci et al. (2014). The successful PCR products were purified, and Sanger sequenced by BioFab Research (Italy, Rome). The sequences obtained were analysed and edited by using Chromas Pro 1.34 and MEGA X v. 11 (Kumar et al., 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis et al., 2008).

3.5. Environmental data (Chapters 2, 3, and 4)

Sea surface temperature (SST; °C) data was obtained from the National Oceanographic and Atmospheric Agency (NOAA) "High-resolution Blended Analysis of Daily SST and Ice" (https://psl.noaa.gov/data/gridded/data.noaa.oisst.v2.highres.html, Huang et al., 2021). These data have a daily frequency and spatial resolution of 0.25° × 0.25° and extend from 1981 to 2021 (inclusive).

Surface chlorophyll concentrations (Chl; $mg \cdot m^{-3}$) were obtained from the NASA (National Aeronautics and Space Administration) Ocean Colour web site (https://oceandata.sci.gsfc.nasa.gov). These data have a monthly frequency, and a spatial resolution of 0.083° × 0.083°. The final data set used corresponds to the MODIS and SEAWIFS sensors and the final time series extend from 1997 to 2021 (both included).

Ocean productivity available to fish (OPFish; %) values (i.e., index that characterises 10–20 % of the global phytoplankton production that effectively fuels higher trophic levels) were obtained from Environmental Marine Information System (2022, April 4).

Information about local atmospheric conditions consisted of a reanalysis of monthly time series of west-east (Ux hereafter) and south-north (Uy) components of the wind (km/h), and precipitation rates (P) from the National Centre for Environmental Prediction/National Centre for Atmospheric Research (NCEP/NCAR, https://psl.noaa.gov/data/gridded/data_ncep.reanalysis.derived.surface.html/, Kalnay et al., 1996). These time series extend from 1948 to 2021 (both included) and have a spatial resolution of 2.5° × 2.5°.

Table 2. Summary of the analytical methods and variables analysed in each of the chapters (Ch.) of this thesis.

	SECTION I	SECTION II			SECTION III	
Variables	**Ch. 1**	**Ch. 2**	**Ch. 3**	**Ch. 4**	**Ch. 5**	**Ch. 6**
Population structure						
Literature review						
Condition						
L_T, W_T						
Kn						
HSI						
Vi						
Tissue fat content						

Mesenteric fat content						
Reproduction						
Visual gonad stage						
Histology						
GSI						
Parasites (ascarioid nematodes)						
UV-press						
Parasitic load indices						
Genetic identification						
Environment						
Chlorophyll						
SST						
OPFish						
Wind						
Precipitation rates						

4. Statistical analyses

Except for the parasite load statistics (**Chapter 5**), which was carried out using Quantitative Parasitology 3.0 software (Reiczigel et al., 2019), developed to manage the left biased frequency distribution of parasites, most of the analyses were performed via R software (http://www.rproject.org/), versions 3.5.1. and 4.2.1 (R Development Core Team, 2018). Statistically significant differences were considered if p-value < 0.05*. Statistical analyses performed in this thesis are summarised in Table 2.

Table 3. Summary of the different statistical methods used in each chapter (Ch.) of this thesis.

Statistical analysis	Purpose of the analysis	Ch. 1	Ch. 2	Ch. 3	Ch. 4	Ch. 5	Ch. 6
Analysis of variance (ANOVA)	To compare mean values of the condition indices between groups. Multi-factor Analysis of Variance was applied to examine the effect of area, season, and reproductive stage on the parameters Bootstrap one-way ANOVA was used to assess the significance of statistical differences in the mean intensity and abundance of larvae (Ch. 5)						

Bootstrap BCa	To adjust both bias and skewness in the bootstrap distribution of the mean intensity of nematodes						
Clopper–Pearson interval	For calculating binomial confidence intervals of the general nematode prevalence						
Dunn's method with Bonferroni adjustment	Post-hoc test applied after a significant Kruskal-Wallis test						
Fisher's exact test	To assess the significance of statistical differences in nematode prevalence						
Games–Howell	Post-hoc test applied after a significant Welch's *t*-test						
Generalized linear model (GLM)	Applied to the indices that did not accomplish with normality and homogenous variances and data transformation was not succeed in correcting the lack of normality or heteroscedasticity						
Kruskall-Wallis	Applied to those parameters in which normal distribution was lacking but homoscedasticity was present						
Levene's test	To check the homogeneity of variances						
Pearson's chi-squared test	To compare among categories of qualitative variables (i.e., mesenteric fat by sex)						
Shapiro-Wilk test	To test the assumption of normality						
Spearman's rank non-parametric correlation test	To explore the relationships between Kn, GSI, HSI, and tissue and mesenteric fat content along with environmental variables (Ch. 2); between the number of nematodes and the sardine parameters (i.e., total length, Kn, GSI, and HSI) (Ch. 5)						
Student's t-distribution	To construct a confidence interval for each mean value of the						

	environmental variables (i.e., SST, Chl, Ux, Uy and P)						
Tukey's range test	Post-hoc test applied after a significant ANOVA						
Two-sample t-test	To test significant differences between two groups (i.e., to compare morphogravimetric parameters between two locations)						
Welch's *t*-test	To compare groups with unequal variances (i.e., GSI by gonadal developmental stage)						

Results

SECTION I

GENETIC VARIABILITY OF SARDINE ALONG ITS DISTRIBUTION

Chapter 1

The current knowledge status of the genetic population structure of the European sardine (*Sardina pilchardus*): uncertainties to be solved for an appropriate fishery management

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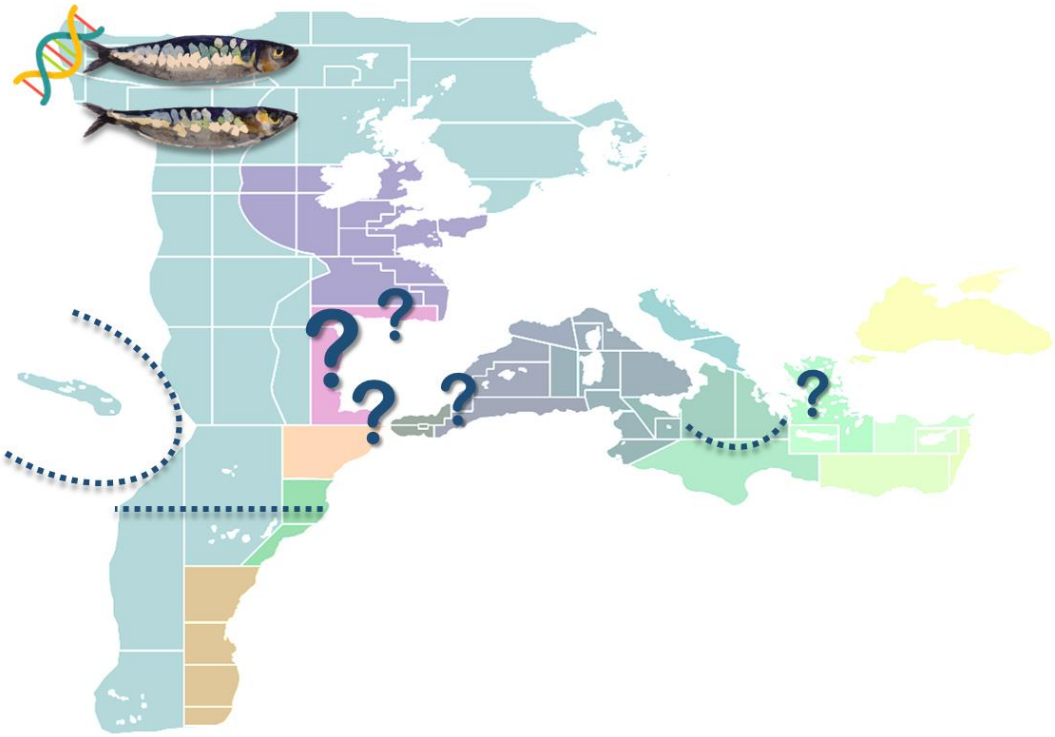

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Abstract

To achieve sustainable fisheries implies that resources' management is carried out in accordance with biologically and ecologically relevant processes. In this context, to infer the boundaries of the genetic stocks along their distribution is crucial to avoid the depletion of genetic diversity induced by fishing pressure. Despite its remarkable ecological role and commercial interest, there are still many uncertainties about the genetic population structure and local adaptation processes of the European sardine (*Sardina pilchardus*) along its distributional range. Our analysis revealed that in addition to the uneven genetic study effort throughout its distribution, there are discrepancies when it comes to delimiting populations, especially in the waters surrounding the Iberian Peninsula. Also, powers of the genetic markers applied in the studies were examined, showing that allozymes detected a larger number of significant pairwise values of genetic differentiation, while mtDNA-RFLP detected a greater degree of differentiation among genetic stocks. Moreover, large values of genetic diversity in all the locations were identified regardless of marker type. Thereby, we provide a discussion of updated knowledge, contributing to shape long-term and genetically sustainable harvest strategies for this pelagic fish, since our findings indicate a mismatch between the genetic stocks and the managed stocks currently defined.

Keywords: Atlantic Ocean • Fish stocks • Local adaptation • Mediterranean Sea • Molecular markers • Pelagic

1.1. Introduction

An essential requirement for sustainable fish resource management is the overlap of biological processes and management action (Reiss et al., 2009). Thus, efforts for matching populations as biological units with management unities or 'fish harvested stocks' have been persecuted in the need to transfer the knowledge between the scientific sector and the fisheries policy and management sectors. Harvested stocks, as fundamental exploited units in fisheries management, are commonly defined based on morphological and demographic characteristics, fishing patterns, connectivity patterns (adult movement and larval dispersal), and more recently also incorporating genetic variability (Huret et al., 2020). In fact, this has been due to the dissatisfaction with performance of phenotypic methods for stock identification, as genetic approaches potentially allow to assess the level of divergent populations that is required to justify a separate management (Waples et al., 2008).

Available information about biology, evolutionary history, ecology and management of the European sardine (*Sardina pilchardus* (Walbaum, 1792)) is still fragmented (Coll & Bellido, 2019), as the delimitation of populations as biological units is still under debate. This may be surprising since S. pilchardus is one of the most important pelagic fish species due to its crucial ecological and economical roles throughout its range of distribution in the North and Central Eastern Atlantic, from the North Sea to the Senegalese coast, including the Mediterranean and the Black Seas (Costalago & Palomera, 2014). Several local studies have been carried out to unravel the genetic stocks of S. pilchardus (e.g., Tinti et al., 2002; Atarhouch et al., 2006; Deli et al., 2020), but only few works cover a greater extension of the sardine's range of distribution (e.g., Antoniou et al., in press; Fonseca et al., in press). In addition, few reviews have tried to compile the information available to provide a general overview of the existing populations (Kasapidis, 2014).

Currently there is no clear consensus on the genetic stock structure of S. pilchardus, which is a common circumstance among pelagic fish taxa. Genetic studies on sardines in the Mediterranean employing different types of markers have reported shallow phylogeographic structure with signs of late Pleistocene expansion, low genetic differentiation and weak or non-existent population structure (Kasapidis, 2014; Antoniou et al., in press). In fact, it was traditionally accepted that coastal pelagic marine fishes show genetic homogeneity and even panmixia (random mating) over their geographic distributions (Moore & Chaplin, 2013). This has to do with several reasons. One is the apparent lack of physical barriers in the marine environment since local patterns of spatial heterogeneity are associated with species diversity patterns (Ayala & Valentine, 1979).

Also, the high dispersal capabilities in larval and adult stages of many marine pelagic species (Magoulas et al., 2006; Bierne et al., 2016) prompts to think about weak genetic differences among conspecific individuals of different areas, complicating population delimitation. Another factor is their important effective population size that limits the impact of genetic drift (Laurent et al., 2007). Finally, and closely linked to the current methodology applied based on molecular markers, it has to be considered that molecular methods could present limitations, since if no genetic divergence is detected among samples, the results are ambiguous and inconclusive. In this context, the causes could be the current gene flow among populations, the belonging to a same genetic stock, or that individuals come from different populations but the method is not able to resolve it due to a lack of resolution of markers (i.e., retention of shared ancestral polymorphisms) (Spanakis et al., 1989; Bossart & Prowell, 1998; Kasapidis, 2014).

This review explores the potential genetic structure of the European sardine by making use of the available data published in the literature. We discuss the different hypotheses raised so far along its distributional range and the selection processes shaping genetic units. We compare methodologies and genetic markers used to date. We also integrate the genetic information available within the context of the present-day fisheries management of this small pelagic fish.

1.2. Methodologies to unravel the genetic population structure of European sardine

Different approaches have been applied to untangle the genetic structure of European sardine populations along its distributional range, even though most studies have been carried out to differentiate genetic units at local level (Table 1).

Table 1. Summary of the genetic population studies of European sardine (*Sardina pilchardus*).

Study	Marker	Area (GSA, FAO)	Comparison	*N*	Ho/h+	He	Genetic differentiation	Evidence of natural selection and/or further conclusions
1 Spanakis et al. (1989)	Allozymes	GSA (20,22)	Aegean vs. Ionian seas	2.6	NA	NA	G = 0.0600 – 27.8600, NS	NO
2 Becheikh et al. (1994)	Allozymes	GSA (12, 13)	Tunisian northern coast (Bizerte)	30	NA	NA	NO	NO
3 Ramon and Castro (1997)	Allozymes	GSA (1, 6, 7)	Spanish Mediterranean coast and Gulf of	45	0.130 ± 0.015	NA	F_{ST} = 0.0749**	North-south cline detected in LDH-1
4 Tinti et al. (2002)	mtDNA-RFLP	GSA (6[a], 17, 18, 19)	Adriatic Sea 1997-1998 vs. Ionian Sea	26	NA	NA	Adriatic-Ionian Φ_{ST} = 0.0047, NS	NO
5 Chlaida et al. (2005)	Allozymes	FAO (27.9.a, 34.1.11,	All samples 2002-2003	35.86	0.317 ± 0.204	0.309 ± 0.192	F_{ST} = 0.2200	NO
6 Atarhouch et al.	mtDNA control region	GSA (3, 6, 22), FAO (27.8.c,	All samples 2002-2004	29	NA	0.997 ± 0.002	Φ_{ST} = 0.0260 (one gene pool); Φ_{ST} =	Isolation and genetic drift acting on the Safi population
7 Laurent et al. (2006)	Allozymes	FAO (27.8.a, 27.8.b,	All samples 2002	47.5	NA	NA	F_{ST} = 0.0050***	Appears to be composed of a single panmictic
8 Atarhouch et al.	Exon-primed intron-crossing	GSA (3), FAO (34.1.11,	All samples 2001 - 2002	49.4	0.39 - 0.58	0.62 - 0.72	F_{ST} = 0.034*	No correlation between geographic and genetic
9 González and Zardoya	Microsatellites	GSA (3, 6, 22), FAO (27.8.c,	Mediterranean vs. Atlantic	47.3	0.747 ± 0.04	0.948 ± 0.00	R_{ST} = 0.0240 ± 0.0260 – 0.0470***	Significant isolation by distance with R_{ST}
10 Laurent et al. (2007)	Allozymes	GSA (3, 7, 22), FAO (27.4.b,	All samples 2003-2004	48.6	NA	NA	F_{ST} = 0.0570**	Selective pressure and isolation by distance of

18 Fonseca et al. (in press)	**17** Antoniou et al. (in	**16** Deli et al. (2020)	**15** Cannas and Tsigenopou	**14** Ruggeri et al. (2012)	**13** Kasapidis et al.	**12** Baibai et al. (2012)	**11** Chlaida et al. (2009)
Whole genome sequence	Genotyping through high-throughput	Allozymes	SNPs	Microsatellites	Microsatellites	Microsatellites and mtDNA control	Allozymes
GSA (1, 6, 13, 17, 22), FAO	GSA (1, 5, 6a, 6b, 6c, 7a, 7b, 9,	GSA (12, 13, 14)	GSA (1, 5, 6, 7, 9, 10, 11, 19),	GSA (17, 18)	GSA (1, 3, 6, 22), FAO (27.4.b,	GSA (1), FAO (27.9.a,	GSA (3), FAO (27.9.a,
All samples	All samples 2017-2018	All samples	All samples 2017-2018	Northern and Southern Adriatic	All samples 1999-2004	All samples Maroccan Coast 2006, Iberian	All samples 2004
6.5	33.16	16.36	35	47.5	73	50	50
NA	0.266 - 0.307	0.1628 ± 0.2244	0.300 ± 0.012	0.765 ± 0.039	0.639 ± 0.114	0.843 ± 0.11	0.048 ± 0.023
NA	0.266 - 0.296	0.1495 ± 0.1883	0.335 ± 0.010	0.795 ± 0.015	0.674 ± 0.119	0.905 ± 0.12	NA
F_{ST} = 0.9600, highest value	F_{ST} = 0 - 0.0408	F_{ST} = 0.1660**	F_{ST} = 0.00174*	θ_{ST} = - 0.0033 to 0.0137	(Sp22);	Θ= 51.505	F_{ST} = 0.2050***
Phylogeographic break between the South of Portugal	Water temperature, and derived environme	NO	NO	NO	Loci (Sp22) appeared to be under selection and	F_{ST} = 0.0017 - 0.0140	Not possible to evaluate the selection with only one locus
				2/45		Isolation by distance between Cap Blanc and	

GSA (Geographical Subarea of the General Fisheries Commission) encompassed within FAO Major Area 37

[+] Ho: Observed heterozygosity for nuclear markers / h: haplotype diversity for mitochondrial markers

NA: Data not reported

[a] Data comparison with other studies

***, ** and * indicate significance at p-value < 0.001, p-value < 0.01 and p-value < 0.05 regarding 'Genetic differentiation'

The first attempts to determine European sardine independent stocks aiming to respect the potential existing populations were based on the study of the phenotype, mainly making use of morphometric and meristic analyses (Regan, 1916; Fage, 1920; Lee, 1961; Silva, 2003). Also, otolith shape indices (Correia et al., 2014; Jemaa et al., 2015) or the biochemical amino acid composition analysis of eye tissue (Riveiro et al., 2011) were applied for discriminating among areas within *Sardina pilchardus* metapopulation. The first approach to estimate the geographical distribution of genetic variability in sardine was allozyme electrophoresis on starch gel applied to samples from the Eastern Mediterranean (Spanakis et al., 1989). Ramon & Castro (1997) also applied electrophoretic methods to characterize stocks of sardines in the Western Mediterranean Sea.

Studies that used genotype-based approaches implying molecular neutral and non-neutral markers can be applied without the need for the complete sardine genome, since haplotype-resolved draft genome of the European sardine has only been recently obtained (Machado et al., 2018; Louro et al., 2019). Among neutral markers, we can find microsatellites (González & Zardoya, 2007; Kasapidis et al., 2012; Ruggeri et al. 2012) and exon-primed intron-crossing PCR (EPIC-PCR), which combines the advantages of microsatellite DNA analysis, including PCR of alcohol-preserved tissue samples as well as high levels of polymorphism, without the need for specific PCR primers (Atarhouch et al., 2007). In the case of non-neutral markers (i.e., those that allow studying the adaptive or evolutionary potential), allozymes (Spanakis et al., 1989; Chlaida et al., 2005, 2009; Laurent & Planes, 2007) or mitochondrial DNA (mtDNA) (Tinti et al., 2002; Atarhouch et al., 2006) have been used. Nowadays, the new technologies allow examining more loci than in the past, permitting the simultaneous study of thousands of loci in hundreds of individuals (Allendorf et al., 2008). The last methodologies applied in sardine studies included genome-wide data set of double-digest RAD-derived SNPs (Coll & Bellido, 2019; Antoniou et al., in press), which can also be applied without complete genome data. Conversely, one of the most recent studies used whole genome sequence data, incorporating also mitochondrial genomes, which enables a comparison between markers with different modes of inheritance (Fonseca et al., in press).

1.3. Genetic variability and population differentiation

Although the studies analysed in this work were developed under different sampling and analytical procedures depending on methodology, we compiled and discussed the available information regarding the genetic variability and population differentiation of *Sardina pilchardus* (Figure 1; Table 1).

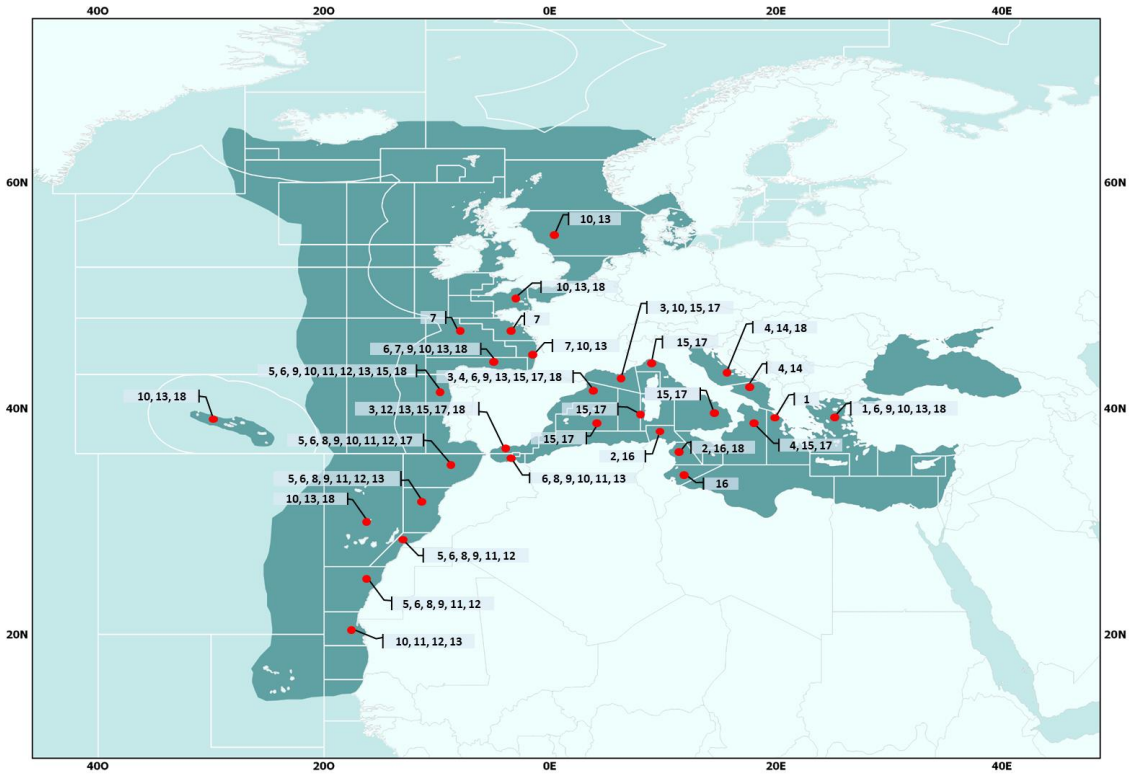


Figure 1. Studies per subareas along the distribution range (darkest blue) of European sardine (*Sardina pilchardus*). Numbers correspond to study numbers in Table 1. Boundaries of FAO Subareas and GSAs (Geographical Subareas) (in FAO Major Fishing Area 37) of the General Fisheries Commission for the Mediterranean are detailed (white lines).

Expected heterozygosity values in studies applying nuclear markers ranged from 0.1495 ± 0.1883 in the Siculo-Tunisian Strait in the analysis performed with allozymes by Deli et al. (2020) to 0.9480 ± 0.0000 in the analysis of González & Zardoya (2007) by applying microsatellites to Atlantic and Mediterranean individuals. Both values are larger than average total heterozygosity ($\overline{H}_T$) and subpopulation heterozygosity ($\overline{H}_S$) observed in marine fishes with allozyme markers, determined as 0.064 and 0.059, respectively, by Ward et al. (1994). On the other hand, mitochondrial markers reach an expected heterozygosity (estimated as haplotype diversity, h) of 0.997 ± 0.002 in the study of Atarhouch et al. (2006), which applied mtDNA control region as genetic marker to mainly untangle the genetic

stocks of sardines from Morocco (coast of Africa), even though it also included other sample areas along the distributional range of S. pilchardus (southern Portugal, the Bay of Biscay, the Catalan Coast and the Aegean Sea) (Table 1).

Many marine species are characterised by large population sizes, high levels of genetic variation and weak differentiation (Ward et al., 1994; Ryman et al., 2014). However, the ratio between effective population size (N_e) and census size (N_c) in these organisms has been estimated to be small, being *S. pilchardus* the species with a lower ratio (of 10^{-8}) of those reviewed by Hauser and Carvalho (2008). In this species, models suggest that high variance in reproductive success can, under some circumstances, produce large effective sizes, while empirical work suggests low effective sizes (Laurent & Planes, 2007; Pinsky & Palumbi, 2014).

Regarding genetic population differentiation, it can be observed in Table 2 that the largest number of significant pairwise F_{ST} or Φ_{ST} values has been identified by the allozymes approach, with a 70.28 % of the comparisons in which genetic differentiation has been detected, followed by the study incorporating EPIC-PCR, with 61.9% of the values with significance. Comparison of significant pairwise F_{ST} or Φ_{ST} data (if nuclear or mitochondrial markers were applied, respectively) among the locations of the studies (Figure 2) revealed that the work that detected greater genetic differentiation values applied mtDNA-restriction fragment length polymorphism (RFLP) marker (differences between Adriatic/Ionian pool and Mediterranean Spanish Coast: $\Phi_{ST} = 0.7414–0.9429$, p-value < 0.0001) (Tinti et al., 2002). This has been supported by the ICES (2017) report, which showed that earlier studies using mtDNA and allozymes indicated a higher degree of differentiation than recent studies using allozymes and studies applying microsatellite DNA for the stocks of European sardine.

Table 2. Summary of the population differentiation significant F_{ST} or Φ_{ST} pairwise values in European sardine (*Sardina pilchardus*).

Marker	Methodology	Number of studies included	Percentage of significant values (%)	Smallest significant value	References
Allozymes	Nuclear	4	70.28 (149/212)	0.0180	3, 5, 11, 16
EPIC-PCR	Nuclear	1	61.9 (13/21)	0.017	8
Microsatellites	Nuclear	3	11.71 (13/111)	0.0080	9, 12, 14
SNPs	Nuclear	1	13.64 (9/66)	0.0209	15
mtDNA control region	Mitochondrial	2	30.77 (20/65)	0.0243	6, 12
mtDNA-RFLP	Mitochondrial	1	16.67 (11/66)	0.7414	4

Pool of the available data. Reference numbers correspond to study numbers in Table 1

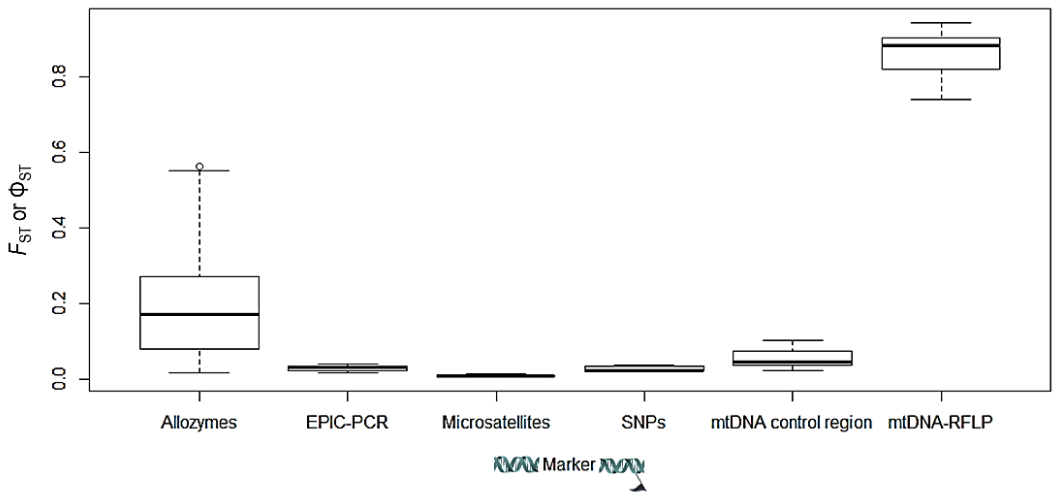


Figure 2. Range of the available significant F_{ST} or Φ_{ST} values in the studies reviewed (Ramon & Castro, 1997; Tinti et al., 2002; Chlaida et al., 2005; Atarhouch et al., 2006; Atarhouch et al., 2007; González & Zardoya 2007; Chlaida et al., 2009; Baibai et al., 2012; Ruggeri et al., 2012; Cannas & Tsigenopoulos 2018; Deli et al., 2020).

By contrast, studies applying microsatellites were the ones that found the least amount of genetic differentiation, as in Ruggeri et al. (2012), which compared two close areas: the northern and the southern Adriatic Sea (GSAs 17 and 18). The analysis of González and Zardoya (2007) also exhibited weak but significant genetic differentiation, in this case, comparing nine locations in the Atlantic Ocean and the Mediterranean Sea using allelic size variation of eight specific microsatellite loci. Conversely, microsatellite loci have been previously described as especially suitable for stock identification in species showing low levels of detectable variation using allozymes or mtDNA, as they exhibit high levels of length mutation resulting in extensive allelic variation and levels of heterozygosity in fish ranging from 24 to 90% (Shaw et al., 1999a, b).

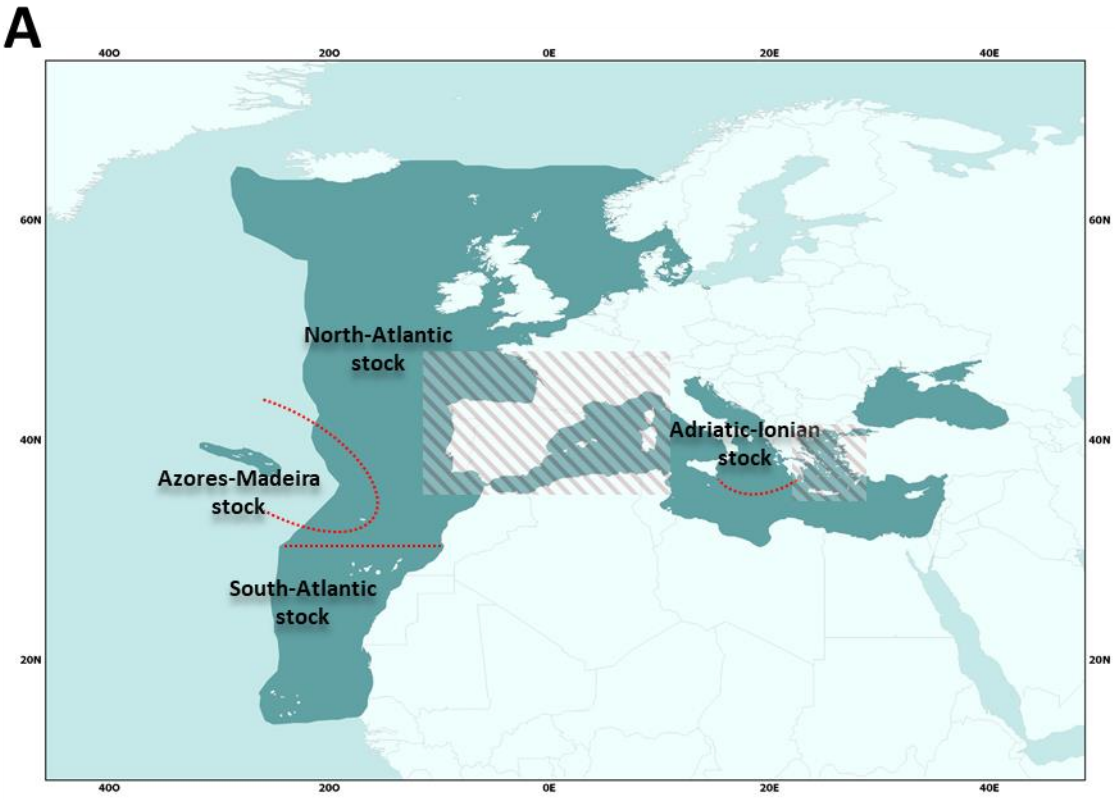
Nevertheless, comparison of the suitability of allozymes, mtDNA, and microsatellites suggested that allozymes and microsatellites are more potent than mtDNA-RFLP studies for the identification of pelagic fish populations. However, molecular markers which appear to be less powerful may still be more appropriate to reveal population divergence under some premises: markers with different modes of inheritance (e.g., nuclear vs. mitochondrial markers), mutation or selection (e.g., allozymes vs. microsatellites) (Hauser & Ward, 1998). This way, in the situation of high levels of gene flow potentially associated with a pelagic species, and therefore low F_{ST}, comparative multi-locus analyses based on both nuclear and mitochondrial genetic markers are probably the most efficient and informative approach to discerning the relative role of historical events and life-history traits in shaping genetic diversity (González & Zardoya, 2007). This combination of markers with different DNA origin is usually based on the use of several (six to eight) microsatellite loci merged with mtDNA sequence, which has been considered the best procedure (Viñas et al., 2011), applied in the study of sardine population structure of Baibai et al. (2012) but incorporating a lower number of microsatellite loci (four). In this context, to fully establish a population structure for sardine, we suggest to incorporate simultaneously two approaches applied to the samples to be compared: mtDNA (or allozymes) markers as non-neutral markers together with neutral markers like microsatellites, which present higher mutation rates (Goldstein et al., 1999). This way, complementary aspects of the evolutionary history of sardine could be showed, with mitochondrial data reflecting past isolation of sardine populations and nuclear DNA data potentially revealing the present gene flow among populations, and a pattern of isolation by distance (González & Zardoya, 2007).

New methods for revealing population structure of European sardine involved whole genome sequencing data, which allow to analyse different parts of the nuclear genome yield population structure and diversity, and also to recover full mitochondrial genomes for comparison with genetic sequences with different modes of inheritance (Fonseca et al., in

press). The algorithm based on multiloci testing of allele frequencies between pairs of samples results in the greatest power to detect more accurately the number of populations (Viñas et al., 2011).

1.4. Mapping sardine genetic population structure

Delimiting genetic population structure of pelagic fish is a difficult task. In general, no genetic structure is evident in most of the distributional range for neutral markers and genetic differentiation among individuals increases as geographical distance increases (ICES, 2017). In fact, results of previous studies have presented inconsistencies and have been non-conclusive determining stock boundaries for European sardine (Figure 3A, B).



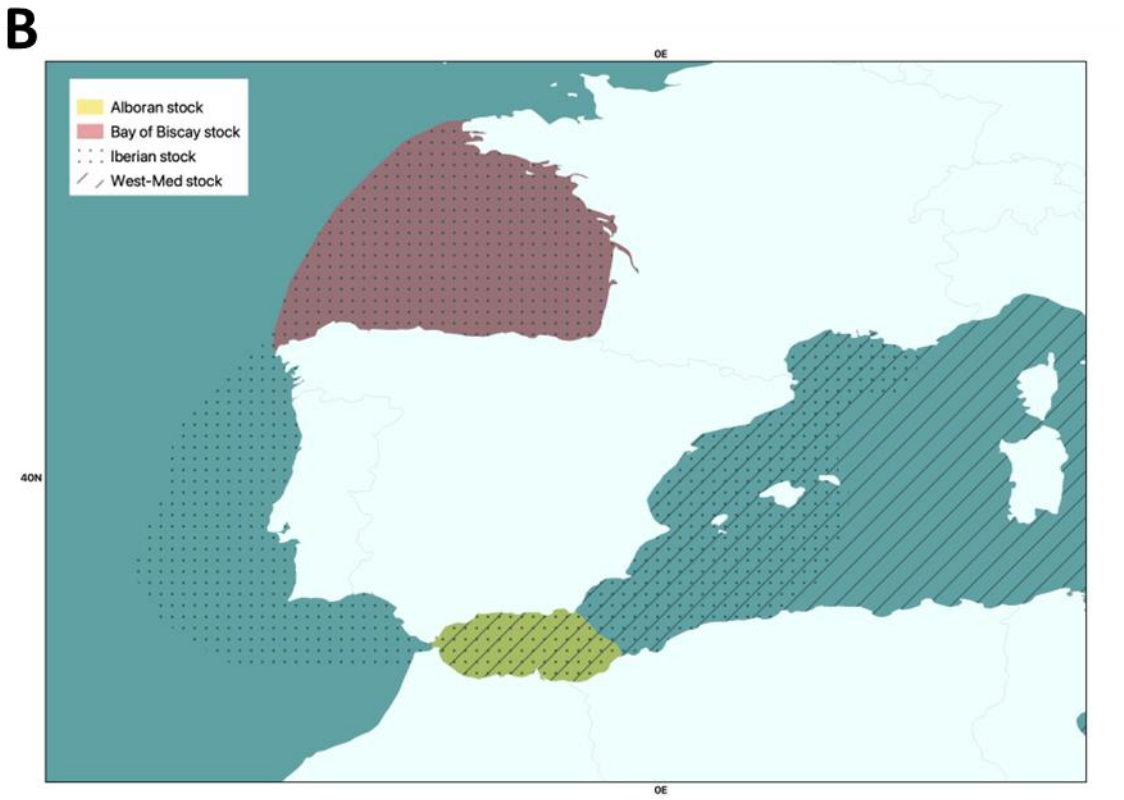


Figure 3. Maps of the European sardine (*Sardina pilchardus*) populations according to the current genetic studies. **A.** Red dotted lines indicate the genetic stocks most robustly defined and probable to date. Diagonal lines represent the areas in which there are more doubts about the definition of the stocks to date. **B.** Different plausible hypotheses according to the authors along the Iberian Peninsula's coasts. Following the investigations compiled in Table 1.

Genetic discontinuities between the North-eastern Atlantic and the Western Mediterranean have been reported or suspected in numerous pelagic and demersal fish species applying allozymes, nuclear and mitochondrial DNA markers (mtDNA) (Naciri et al., 1999; Comesaña et al., 2008). In previous decades, some authors have considered the Mediterranean sardine as an independent subspecies of the Atlantic sardine regarding morphometric and meristic characteristics (Regan, 1916; Lee, 1961): *S. p. pilchardus* on the Atlantic coast of Portugal, and *S. p. sardina* in the Mediterranean and on the Atlantic coast of Africa. Nevertheless, it has not been supported by most genetic analyses (González & Zardoya, 2007; Kasapidis et al., 2012), despite some results that back this structure (Atarhouch et al., 2006). It has to be taken into account that, in general, fish species have greater variance in morphological traits both within and among populations than do other vertebrates, being more susceptible to environmentally-induced morphological divergence (Kinsey et al., 1994), so that the molecular analysis is of special importance at the

ichthyological level. A clear example in sardine was reported by Antoniou et al. (in press), who described those individuals of the Mediterranean and the Atlantic respond differently to environmental pressures.

Using the mtDNA control region, Atarhouch et al. (2006) distinguished only the population of the Bay of Biscay from the rest of both Atlantic and Mediterranean populations, unlike other studies that have not found genetic differentiation among sardines in Celtic Seas, English Channel and the Bay of Biscay (ICES 2017). In fact, Laurent et al. (2006) determined that it appears to shape a single panmictic population despite evidence of a gradual change in two loci. Some investigations did not even identify genetic differences among the Bay of Biscay and the Western Mediterranean samples of European sardine (Kasapidis et al., 2012; Fonseca et al., in press). Moreover, preliminary results after applying genotyping through high-throughput showed that the most important component of genetic divergence among populations occurs between the Atlantic and the Western Mediterranean Sea, whereas the Alboran Sea samples are closer to the Atlantic than the Mediterranean samples (Coll & Bellido, 2019; Antoniou et al., in press). Preliminary results of Fonseca et al. (in press) after low coverage whole genome sequencing show three genetic clusters: one including individuals from Azores and Madeira, the second corresponding to the whole perimeter of the Iberian Peninsula, involving the Bay of Biscay as well as the Alboran and the Catalan Coast, and the third including the Eastern Mediterranean samples and those from the Canary Islands. Kasapidis et al. (2012) also detected a genetic heterogeneity among the sardine stocks of Madeira and the Azores, and the rest of the Atlantic individuals assessed by using five microsatellite loci.

Some of the most relevant sardine population studies on the southern half of the Eastern Atlantic distribution are those of Laurent et al. (2007) and Chlaida et al. (2005, 2009) performed with allozymes. These authors identified genetic homogeneity, even among populations of northern Morocco and southern Portugal, except for the superoxide dismutase (SOD) locus, whose allelic distribution follows an isolation by distance model. Based on their results, two fishing stocks are proposed on the Eastern Atlantic coast: one to the south of the Bay of Agadir, and other to the north, which seems to be genetically related to Mediterranean populations. All these observations were confirmed by Atarhouch et al. (2007) based on the intron polymorphism of two nuclear genes (CaM-4 and Ops-1), although this study defined as weak the genetic boundary in the Bay of Agadir. In addition, Baibai et al. (2012), who combined microsatellites and mtDNA control region markers, supported genetic differentiation between the sardine population of the Atlantic Moroccan coast and the Spanish coast of Galicia, with a potential contact zone around Cádiz in the

Atlantic coast and Málaga for Mediterranean Sea, revealed by the large number of polymorphic sites of the latter.

Local studies in the Mediterranean performed with allozymes described differences between the populations across the Siculo-Tunisian Strait, with the presence of chaotic and complex genetic patchiness (Deli et al., 2020). Besides, different genetic stocks were delimitated in the Aegean and Ionian Seas, despite the fact that for some characters the within-area variation was larger than the between-area variation (Spanakis et al., 1989). Kasapidis et al. (2012) also detected differences regarding the Aegean individuals with respect of the Atlantic and other Mediterranean samples by using microsatellite markers, except for samples from Northern Spain (Catalan Coast), thus being grouped into the same genetic stock by the authors.

In addition, Tinti et al. (2002) detected a genetic homogeneity in the distribution of cytochrome b haplotypes between the Adriatic and Ionian populations and clear differences of these with respect to the Mediterranean Iberian populations. Furthermore, Ramon & Castro (1997) and Kasapidis et al. (2012) described population structure along the west coast of the Mediterranean, with notable differences between the population in the Alboran Sea and the rest of the Mediterranean, contradicted by Fonseca et al. (in press).

1.5. Selection processes and drivers shaping European sardine's genetic units

Traditionally, population structure in marine species has been explained by' isolation by distance' in an extended way, which may occur when the distribution of the organisms is larger than the dispersal range of individuals (Wright, 1943; Bekkevold et al., 2005). However, marine organisms are permanently exposed to physical–chemical variables as temperature or salinity, among others, as well as to biological agents like parasitism, and to anthropogenic factors such as pollutants, human-induced global warming, or fisheries. The latter contributes to catch-induced selection based on traits related to maturation and growth, eroding functional genetic diversity (Marty et al., 2015), which may be especially relevant to overexploited *Sardina pilchardus*. All the mentioned factors may cause divergent selection by means of differential survival of animals with different genotypes in distinct environment (Kasapidis et al., 2012). On the other hand, oceanographic currents, topography of the ocean floor (Bekkevold et al., 2005), and other physical and hydrological

barriers typical in straits (e.g., Gibraltar) could limit gene flow, contributing to population differentiation.

In addition, intrinsic characteristics of the individuals comprising the population as different habitat preferences and behaviour (i.e., homing or habitat choice), sexual selection or limited dispersal capabilities are also shapers of genetic heterogeneity (Faria et al., 2021; Fonseca et al., in press). In fact, even though clupeids are commonly considered highly mobile, their localized spawning behaviour or migratory patterns may result in restricted gene flow between populations (Bacha et al., 2014).

Thereby, to develop and use molecular markers that allow characterising non-neutral genomic regions responding to adaptive variation is gaining value, especially among marine populations, to which erroneous homogeneity has been attributed. This relatively recent ability to identify DNA regions and genes under the influence of selection is closing the gap between molecular scientists and researchers who are interested in addressing the role of local adaptation in shaping biodiversity (Kirk & Freeland 2011). One example of analysis to decipher selection is the assay of allelic frequencies of the allozyme SOD, which has previously been used in S. pilchardus to discriminate fish stocks (Ramon & Castro 1997; Chlaida et al., 2005; Laurent et al., 2007; Chlaida et al., 2009). However, Chlaida et al. (2009) refused the idea that SOD locus was under selection throughout the Atlantic and Mediterranean Moroccan coastlines. By contrast, Laurent et al. (2007) suggested that genetic analysis of S. pilchardus over 15 locations between the North Sea and Mauritania, including samples from the Azores, Madeira, and the Mediterranean Sea, showed both isolation by distance and local selection on the single locus SOD, which may be under natural selection pressure with abrupt changes associated with a hydrodynamic barrier in the Gulf of Agadir (Chlaida et al., 2009). Baibai et al. (2012) also identified genetic differentiation following an isolation by distance pattern along the Moroccan Atlantic coast, in addition to some degree of isolation of the northern and southernmost stocks analysed (Galician and Cap Blac, respectively). This was potentially associated with oceanographic barriers (e.g., gyres) or environmental barriers like the presence of several emergence of upwelling in the south of Morocco. A recent study suggested that environmental variables are crucial at all levels of population structuring between populations of the Atlantic (Gulf of Cadiz) and Western Mediterranean and within populations of the Western Mediterranean Sea (Antoniou et al., in press), in particular the ones related to sea surface temperature.

Focusing on the Atlantic genetic heterogeneity, the cluster Azores-Madeira was defined by Kasapidis et al. (2012), as well as by Fonseca et al. (in press) (Figure 3A). The genetic divergence between the stock of Azores-Madeira ecoregion compared to stocks in

other Atlantic-Mediterranean regions may be the result of genetic drift due to isolation during the Pleistocene glaciations, the absence of suitable corridors for migrants between the islands and continent, and/or the disruptive effect of the Portugal current, flowing along the East Atlantic coast from northern Portugal southwards to northern Africa (Domingues et al., 2007; Pérez-Portela et al., 2017). Here we should add the effects of other current systems as the Azores and the Canary Currents (Sá-Pinto et al., 2008) or the deep oceanic waters that separate stocks from those of the continental shelf (Kasapidis et al., 2012). These oceanic domains also define to a large extent groups of populations that are genetically distinct from neighbouring population groups in other ocean domains (Magoulas et al., 2006).

On the other hand, and focusing on the genetic Mediterranean stocks, Agostini & Bakun (2002) described five sub-basins based on nutrient enrichment, concentration of larval food distributions, and local retention of eggs and larvae, which could be causing differentiation of clupeoid fish stocks at a genetic level: the Alboran Sea, the Adriatic Sea, the Aegean Sea, the Gulf of Lion and nearby Catalan Coast, and the Straits of Sicily/Tunisian Coast. As it seems, four of these zones overlap with the potential European sardine populations defined in the literature (Figure 3A, B).

The Almeria-Oran Front, a well-defined oceanographic break situated east of the Strait of Gibraltar, is described as responsible for hindering gene flow between Mediterranean and Atlantic fish populations of many fish species (Schunter et al., 2011; Fonseca et al., in press), being the major oceanographic discontinuity in the Western Mediterranean (Naciri et al., 1999). However, hydrological characteristics of the Alboran Sea's surface waters are much closer to those of the North-eastern Atlantic than the Western Mediterranean (Bacha et al., 2014), which matches the similarity between Alboran Sea samples and the Atlantic samples compared to the Mediterranean samples (Coll & Bellido 2019; Antoniou et al., in press), and also supported by hints of gene flow between Alboran and Moroccan Atlantic Ocean coast stocks (Baibai et al., 2012). Studies reported sardine nursery areas within the Bay of Málaga (Würtz, 2010; Quintanilla et al., 2020), located in the central part of the northern Alboran coastline, which provides shelter from the large-scale westerly wind flow (Würtz 2010). Besides, the presence of the Alboran gyre could be causing larvae retention (Naciri et al. 1999) and hindering dispersal and migration (Bacha et al. 2014) of the juveniles and reproductively active adults. However, the genetic break inferred with respect to other Atlantic and Mediterranean stocks may include not only several oceanographic discontinuities but also genetic differentiation because of isolation by distance (Schunter et al. 2011).

Topographic features as continental shelf-breaks, peninsulas and capes, and enclosed gulfs promote the retention of eggs and larvae of clupeids in the productive spawning areas like the shelf-break front in the Western Mediterranean, the land enclosure of the Adriatic Sea and the North Aegean Sea (Somarakis et al., 2019). Genetic differentiation linked to larval retention and confined dispersal is probable in the Adriatic and Aegean Seas, since the peninsulas partially isolate their waters from the rest of the Mediterranean (Magoulas et al., 2006), reducing genetical flow toward other basins. To the south of each of these basins there are mesoscale anticyclonic eddies produced by unstable parts of the gyres (Würtz, 2010), which may further enhance the genetic isolation of sardine populations, confirming the results of Spanakis et al. (1989) and Kasapidis et al. (2012).

After what has been discussed, it could be expected that in the Eastern Atlantic, the distribution of European sardine is mostly driven by the phenomenon of isolation by distance since such an extensive body of water shows fewer physical barriers associated with coastal relief, although it should not be forgotten that oceanographic elements such as currents, gyres, upwelling, and oceanic fronts produce physically heterogeneous seascapes that may be obstacles to gene flow and, therefore, may contribute to population differentiation (e.g., a potential explanation for Azores-Madeira genetic stock). On the contrary, in the Mediterranean Sea the selection processes based mainly on physical and hydrological barriers could be potentially shaping the sardine genetic stocks. Homogeneity of Atlantic population groups has been shown in other pelagic and demersal species (Recasens et al., 1998; Souche et al., 2015), with the existence of single large panmictic units. However, in a semi-enclosed Mediterranean with sub-basins, there is likely a greater genetic heterogeneity regarding sardine stocks. In fact, it is considered as a hotspot for fish endemism (Lasram & Mouillot, 2009; Coll et al., 2010) characterized by relatively high biodiversity due to its geological evolution and environmental conditions (Granger et al., 2015). This may be the reason why the efforts and discrepancies in the limitations of sardine genetic units are potentially greater in this region. Likewise, it has been observed that a common mapping of the stocks present on the coasts of the Iberian Peninsula remains to be established (Figure 3B), which represents a transition between the Atlantic and Mediterranean waters. Despite a wealth of historical and oceanographic data, in the Atlantic–Mediterranean transition there are still discordant results regarding the biogeographical separation for many species (Patarnello et al., 2007).

To gauge the importance of the different variables influencing genetic structure in a changing environment due to the global change, it is imperative to exhaustively evaluate the impact that the factors previously mentioned have in S. pilchardus populations adaptation and, therefore, potential differentiation. This can be helpful not only for the current

managing of the biological resource but also to project and develop scenarios taking into consideration genetic units and future directions of change.

1.6. Spatial mismatch between biological and fishing management units: implications

Although most of the concern in the literature about genetic changes caused by exploitation has focused on marine and freshwater finfish populations (Allendorf et al., 2008), there are large unknowns about the genetic consequences of harvesting these taxa, partly because temporal investigations that evaluate phenotypic and molecular changes simultaneously are scarce (Pukk et al., 2013). However, it is clear that intense fishing pressures may induce neutral and adaptive evolution affecting life-history traits, with a negative impact on the genetic diversity of exploited populations through selection and genetic drift (Marty et al., 2015; Pavičić et al., 2020). Thereby, with the aim of minimizing these effects and protecting genetic diversity, stock structure and population connectivity data are vital for effective fisheries management (Pavičić et al., 2020).

In EU Atlantic waters, two sardine stocks are considered (Figure 4): Northern stock (ICES Subareas 27.7 and 27.8.a, b, d) fished mainly by France and Spain, and Southern stock (ICES Subareas 27.8.c and 27.9.a) fished by Spain and Portugal (ICES 2017). In this sense, and mainly due to the existing doubts regarding the genetic stocks along the Iberian Coast, it is unknown whether it is necessary to separate these two stocks, or whether they belong to the same genetic pool. Nevertheless, if limits are established for fisheries management in this area, current studies point to the subareas that comprise the Bay of Biscay (ICES Subareas 27.8.a, b, c, d) as a single distinct unit for this species. In addition, it is not clear if there could be an 'Iberian' genetic stock, in which sardines in the Bay of Biscay and the Western Mediterranean are genetically similar, which would cause the need to develop a joint management of Atlantic and Western Mediterranean despite potential practical limitations. On the other hand, there is a large part of sardine distribution (e.g., FAO subareas 27.3, 4, 5, 6) in which its management is not mentioned because sardine as a fishing resource is hardly exploited in these areas.

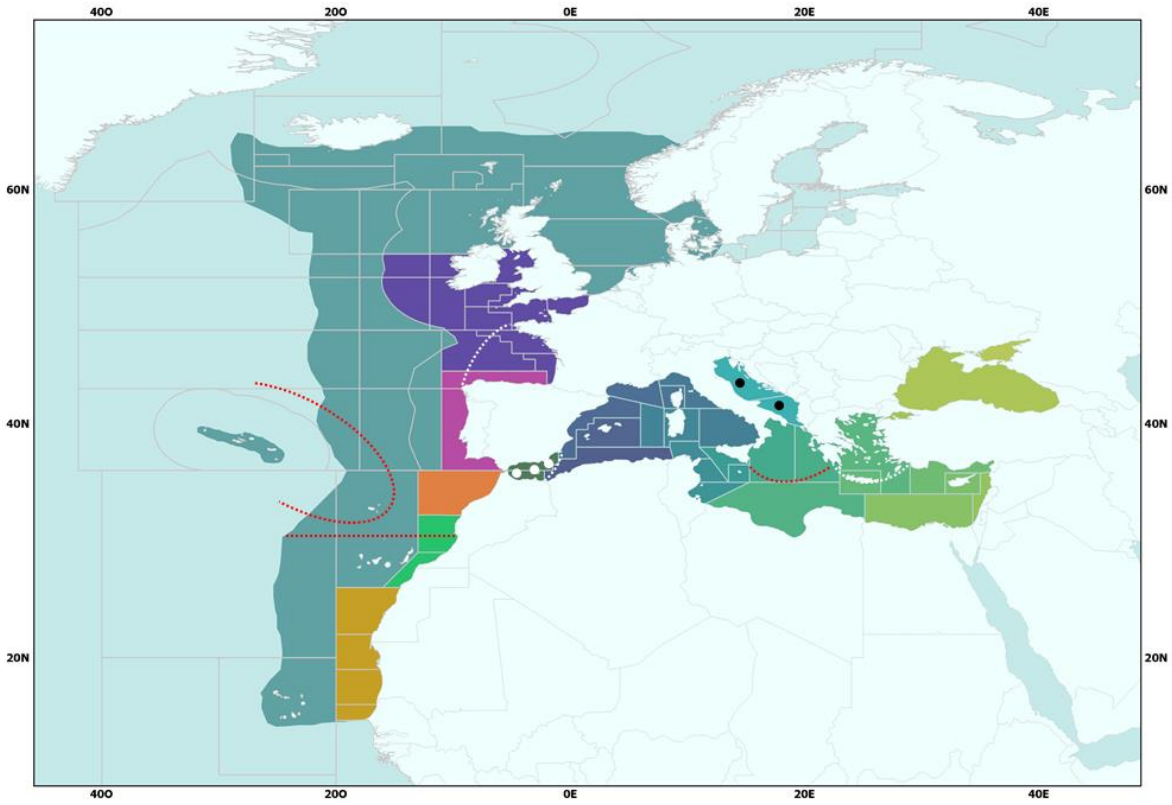


Figure 4. Map of the potential genetic stocks of the European sardine together with the present-day defined fishing stocks. Dotted lines indicate the genetic stocks determined by the current works (red-dotted lines: robust genetic stocks; white-dotted lines: potential genetic stocks). Colours indicate the current management stocks: purple: Northern European Stock; pink: Southern European Stock; red: Northern African Stock; green: Central African Stock; orange: Southern African Stock; blue-turquoise transition shades along the Mediterranean Sea: Mediterranean Stocks managed by GSA [Validated stock assessment forms (SAFs)]. Spots represent shared management for multiannual plans [white spots: Alboran area (GSAs 1, 2, 3); black spots: Adriatic area (GSAs 17, 18)].

Also, FAO has delimited four fishing zones with the aim of managing the sardine off the Northwest African coast: Northern Zone: 35° 45′ N–32° N (Cape Spartel-Eljadida); Zone A: 32° N–29° N (Safi-Sidi Ifni); Zone B: 29° N–26° N (Sidi Ifni-Cape Bojador); Zone C: 26° N-Southwards (Cape Bojador-the southern extent of the species). However, working groups of FAO chose to adopt three separate stocks: Northern Stock (35° 45′ N–32° N); Central Stock (32° N–26° N) (Zones A + B); Southern Stock (26° N-the southern extent of the species) (Zone C), with an evaluation limited to two distinct stocks (Zones A + B and Zone C) (FAO, 2001). Taking into consideration the genetic stock divisions in accordance with current knowledge, the most appropriate local management approach in African waters should be based on establishing a Northern and Southern African stocks, delimited by the Bay of Agadir (30° 48′ N) (Figure 4). However, the delimitation by this barrier to gene flow

could represent the boundary between two stocks at the Atlantic level in the sardine distribution range: a Northern and a Southern Atlantic stocks.

The potential genetic stock of Azores-Madeira defined by Kasapidis et al. (2012) and Fonseca et al. (in press) would currently be out of an independent and specific management. In this context and under the precautionary approach, any partly conflicting conclusions in the matter of the genetic population structure of a species must not prevent management being developed in relation to the potential underlying structure of populations (Reiss et al., 2009) to avoid the loss of genetic variation. Although diversity can be rebuilt through mutation or through immigration from genetic refugia, ignoring differentiated populations in harvest management could hinder genetic diversity recovery, as when an entire species is considered one stock, there are no potential refugia (Pinsky & Palumbi, 2014).

Focusing on the Mediterranean, scientific advice of sardine fisheries (Working Group on Stock Assessment of Small Pelagic Species) in the framework of the General Fisheries Commission for the Mediterranean (GFCM) has used the geographical subareas (GSAs) as differentiated stocks to diagnose its status as well as to provide advice and recommendations for a better management (FAO, 2019). The exceptions are the Alboran (GSAs 1, 2, 3) and Adriatic (GSAs 17, 18) stocks, each of which have been consider as single units in which sardine stocks are shared (Fiorentino & Vitale, 2021). As we have seen, there is no consensus among studies regarding the genetic heterogeneity in this semi-enclosed waterbody, even though some studies coincide in separating an Adriatic-Ionian stock from the rest of the Mediterranean (Tinti et al., 2002; Kasapidis et al., 2012), which also happens with Alboran individuals (Ramon & Castro, 1997; Kasapidis et al., 2012). On the last case, the management unit would potentially fit with the genetic unit. However, in the former, a joint management between GSAs 17, 18 (Adriatic) and GSAs 19, 20 (and maybe, 21) (Ionian) should be considered. Likewise, there is the question of the possible existence of a genetic stock in the Aegean, so following this precautionary principle (Reiss et al., 2009), its independent management could be proving appropriate. Thus, all seems to indicate that more assessment and management units have been established than sardine genetic stocks in the Mediterranean. Misspecifying spatial population structure and migration by the fishery can produce large assessment errors when stock assessment is conducted based on management areas (Guan et al., 2013). Thus, the overestimation of the number of stocks of a species could lead to biases in estimates of fishing mortality and yield, generating the erroneous idea of a more intensive exploitation of a healthier population that actually spreads across several of the management areas, and transferring that pressure to another genetic stock that is actually in worse condition, or misinterpreting the dynamics of areas where the species has been historically depleted (e.g., the case of the Gulf of Lion (GSA 7)

and the Catalan Coast (GSA 6). The focus was on the drop in sardine abundance of the former stock (Antoniou et al., in press), although it has been observed that there is genetic homogeneity with respect to individuals from the Catalan Coast (Coll & Bellido, 2019)).

1.7. Discussion and final conclusions

We showed the difficulty of studying the population structure of a pelagic species. As we have discussed, there are currently gaps of information about the population structure of European sardine. One of the most important unsolved questions is that it is not clear if there are two sardine subspecies (i.e., *S. p. pilchardus* and *S. p. sardina*), although recent genetic studies have rejected this possibility.

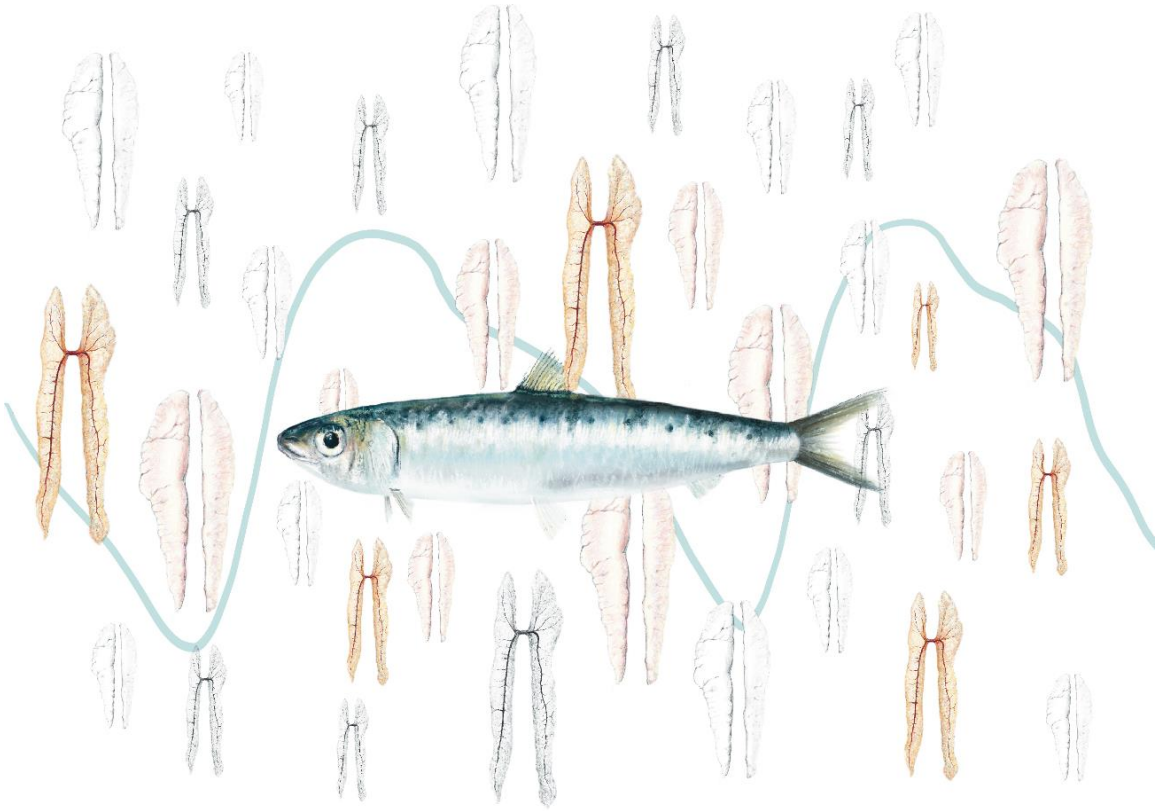
Besides, disparate hypotheses of stock structure have emerged around the Iberian Peninsula and the Aegean Sea. In addition, current methods have limitations, since if homogeneity between stocks is detected, it may be due to real genetic flow or due to a lack of statistical power of the genetic marker, incurring a type II error (i.e., the non-rejection of a false null hypothesis). Counterintuitively, mtDNA-RFLP marker showed higher genetic differentiation values than other markers as microsatellites or allozymes, even though the latter detected a larger number of significant pairwise values of genetic differentiation. Thereby, we suggest the combination of several approaches involving nuclear and mitochondrial information to increase the power of detecting population differences. On the other hand, genetic diversity values among studies were miscellaneous, probably because of the results depend on the areas to be compared in addition to the sample size and the techniques used in each investigation. However, these estimates of diversity were larger than expected in marine fish species. The study of the loss of genetic variation is especially of interest in pelagic fishes, as it can occur when census population sizes are large because the genetically effective population size is often much smaller than the census size (Allendorf et al., 2008), as highlighted in the case of sardine (Laurent & Planes, 2007; Pinsky & Palumbi, 2014).

Another conclusion is the uneven study effort along the distribution of *S. pilchardus*. Genetic sampling of sardine specimens along the Black Sea, the Southern Ionian Sea and the Levantine Sea, as well as the northern and the southernmost Atlantic distribution is scarce or non-existent. By contrast, it is one of the most caught species in the Mediterranean eastern basin (i.e., Levantine Sea) (Marttin et al., 2006; Eurostat, 2008), although that does not occur in the northernmost East Atlantic Ocean, where the target fishes with higher

economical value are different. Both the divergence in determining the spatial limits of each genetic stock among the reviewed works and the paucity of studies due to a biased sampling effort have direct implications on fisheries management. Thus, further studies are required to re-evaluate and determine the population structure of the European sardine in order to apply a differential fisheries management approach of sardine genetic stocks. Fishing management areas are not updated based on the current knowledge of the genetic structure and there are areas in which the number of biological units has been overestimated (e.g., the African coast and the Western Mediterranean Sea) and areas that are not managed independently even though they seem to be differentiated entities (i.e., Madeira-Azores).

SECTION II

SPATIAL VARIABILITY IN SARDINE CONDITION AND REPRODUCTION



Chapter 2

Somatic condition and reproductive potential as a tandem in European sardine: an analysis with environmental perspective in the Northern Adriatic (Gulf of Trieste)

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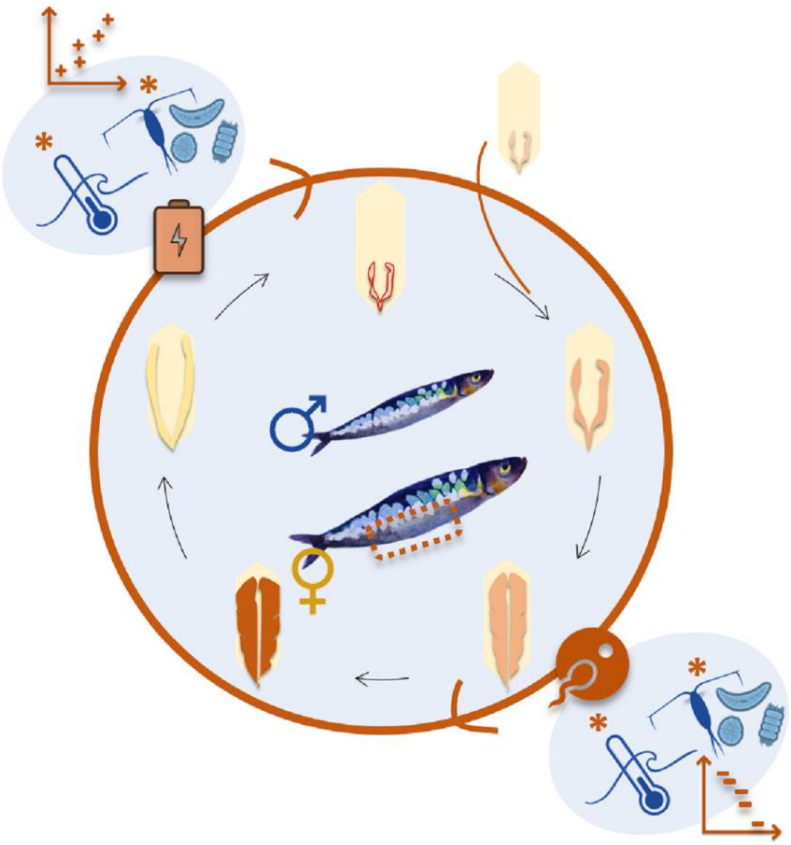

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Abstract

The European sardine's condition is reflected in its reproductive potential, and therefore, in its status as a fishery resource. These values depend on the stock's distribution and resource availability, which is highly determined by environmental characteristics. Sardines from the productive Gulf of Trieste (in the North Adriatic), located in the northernmost section of the most septentrional Mediterranean sub-basin in which sardine exploitation has traditionally been intensive, were analysed. The reproductive cycle and gonadosomatic index (GSI) were studied. Tissue and mesenteric fat values, as well as vacuity (% Vi), relative condition (Kn), and hepatosomatic (HSI) indices were evaluated due to their potential relationships with reproductive performance. The results suggested opposite patterns between fat reserves and GSI, while Kn showed a relationship neither with GSI, nor with reproductive stage, which led us to conclude that it is more advisable to apply direct lipid indices to project their contribution to reproductive potential. Moreover, the females' condition was generally better than that of the males, added to an advanced gonadal development during spring and summer, albeit males and females reached the spawning season together. Moreover, females' GSIs were significantly higher during active spawning. Furthermore, correlation analyses showed that SST was related with the parameters evaluated, as well as the available portion of productivity for the fish (OPFish), which may explain the sardines' better condition and GSIs than their chlorophyll concentration.

Keywords: Fat content • Mediterranean • *Sardina pilchardus* • Reproduction • OPFish • Pelagic

2.1. Introduction

The reproductive potential of fish is defined as the capacity of a fish stock to produce gametes and viable offspring (Marteinsdottir & Begg, 2002; Ganias et al., 2014). Thus, it is a main aspect in fish conservation, especially when the stock is within the sustainable limits of fish exploitation, or when they are overexploited. Small and medium sized pelagic fish are characterised by short life cycles and small body size, and so early maturation, many eggs per body mass, and batch spawning are common strategies to compensate for their short lifetime fecundity (Ganias et al., 2014). There is a wide range of evaluations of the reproductive potential in fish, with both qualitative and quantitative approaches that allow us to infer their fecundity and laying season. However, especially in the above-mentioned species, the evolution of their condition and lipid reserves must be taken into account to make an exhaustive analysis of their reproductive potential. In fact, reproductive timing, potential and batch fecundity, and egg quality are related to females' size and condition in capital breeders (Alonso-Fernández, 2011), and so both general condition and energy storage have important implications for recruitment and pelagic ecosystem structures (Albo-Puigserver et al., 2017; Saraux et al., 2019; Albo-Puigserver et al., 2020). Thus, condition should be carefully studied to be included in assessment models (Domínguez-Petit & Saborido-Rey, (2010) or models that infer reproductive potential (Marteinsdottir & Begg, 2002) to an effective management of the fishing resource.

The reproductive potential of the small pelagic European sardine (*Sardina pilchardus* (Walbaum, 1792)) depends on its spawning frequency and batch fecundity (Kim et al., 2013), as it is a multiple spawner species with an indeterminate annual fecundity that serially releases batches of pelagic eggs at intervals (i.e., batch spawner) within the spawning season (Zwolinski et al., 2001; Ganias et al., 2004). Its spawning season occurs during the winter (Ganias et al., 2014; Tsikliras & Koutrakis, 2013) along its distributional range, although monthly differences exist associated with sardine stock genetic features and environmental characteristics of the area (Basilone et al., 2021). Production of such a great number of eggs during an extended period requires a considerable amount of energy resources that can be obtained (1) from energy reserves accumulated prior to spawning, (2) directly from food input during the spawning season, or (3) from both sources (Nunes et al., 2011) so that the population's reproductive potential can be influenced by the condition of the fish, which has a direct impact on their recruitment strength (Kim et al., 2013). In fact, during the reproductive period, the relative lipid content destined to gonad growth and development may gradually become even more important than growth in length (Machias & Tsimenides, 1995). The European sardine presents a cold-temperate

water affinity, and it is distributed throughout the northeast of the Atlantic Ocean, and from the North Sea to Senegal, the Sea of Marmara, the Black Sea, and the Mediterranean Sea (Parrish et al., 1989). It could be considered one of the most important species within the Eastern Central Atlantic and the Mediterranean Sea not only because of its crucial intermediate trophic level for the ecosystem as a filter feeder (Cury et al., 2000; Van Beveren et al., 2014), but also as a human font of essential lipids rich in long-chain polyunsaturated fatty acids (PUFA) and its high-value, easy digestible proteins which contain all essential amino acids necessary for healthy human diets, along with minerals and vitamins (Šimat et al., 2020). Thus, it represents a great number of catches, being the main contributor to total landings for the whole Mediterranean Sea, together with the anchovy (Lleonart & Maynou, 2003). Therefore, when added to other environmental pressures, a great fishing pressure has caused the biomass of many stocks to be below biologically sustainable levels (FAO, 2020), with a compromised health status. In this sea, its importance could be highlighted, especially in the Adriatic, where sardines represent 41 % of total marine catches (Morello & Arneri, 2009; Pacetti et al., 2013). Sardine fishing covers a great part of the Adriatic basin, but it is mostly concentrated in the northern and central parts in which Italy's main catches come from (between Trieste and Vieste) (Morello & Arneri, 2009; Carpi et al., 2017). In this area, a decline in sardine body condition has been observed over the last two decades (Brosset et al., 2017), and reports have documented the sardine's poor state over a large period of time (Popescu, 2018).

Sardines primarily feed on small species of zooplankton (copepods, decapods, cirripedes, fish eggs, and cladocerans) and phytoplankton (diatoms and dinoflagellates) whose contribution to individuals' diets varies depending on fish length, season, and region considered (Bertrand et al., 2022). In general terms, lipids are the preferred source of metabolic energy for growth, movement, and reproduction in pelagic fish, and are the first macro-molecules to be catabolised, and so the measurement of lipid content has been preferably used in the study of small pelagic fish conditions, including sardines (Albo-Puigserver et al., 2020). Sardine lipid content varies widely with season, which has a direct effect on food availability, water temperature, and, ultimately, the sexual state of the animal (Bandarra et al., 1997). An energy surplus to the essential standard metabolic requirements (i.e., maintenance, locomotion, predation avoidance, and feeding activity) is allocated to somatic growth, energy storage, or reproduction after the individual reaches sexual maturation, and that is why it can be stated that fish reproductive investment is the result of essential life history trade-offs in resource allocation (Nunes et al., 2011).

Therefore, any factor influencing the energy transfer to gonadal development may have an effect on the reproduction of the individual. Both ovarian maturation and fish fecundity are linked to energy reserves, and hence food supply, since energy availability can cause variations in egg production, with likely impacts on ovarian allometry (i.e., the extent of gonadal growth and development) (Somarakis et al., 2004). Moreover, the fish endocrine system is modulated by external and internal (size and/or age, storage levels, i.e., levels of sugars, amino acids, and lipids) conditions, and thus it can either complete reproductive development and spawning under an optimal environment or delay/abort reproduction under non-optimal circumstances (Volkoff & London, 2018). Therefore, there exists high variability in terms of fish recruitment, biomass, and distribution, which are mostly dependent on environmental and climatic conditions (Basilone et al., 2021). In fact, it has been reported that higher catches and larger larval growth rates are found in areas with high chlorophyll concentrations (Vargas-Yáñez et al., 2020), with water stratification and currents among the most important factors that modulate plankton productivity and availability and, therewith, better condition of the planktivorous fish (Brosset et al., 2017). Further, it should be highlighted that among the variables that could have an effect on this process, water temperatures of < 18 °C have been documented to allow sardines to reach maximum spawning activity (Catalán et al., 2006).

Under the current changing environment, knowledge of the energetic and reproductive physiology of marine resources has gained even more importance, since both can be compromised by the additional environmental pressures linked to this phenomenon, hindering their management inevitably. In a climate change scenario, marine heatwaves have been longer and more frequent during the last century (Oliver et al., 2018), and discharge rates from the relationships between evapotranspiration, precipitation, and river flows during extreme wet and dry years are turning out to be much wider (Viaroli et al., 2015), causing changes in productivity due to the alteration of the distribution and availability of nutrients (Guisande et al., 2004; Brown et al., 2010). Moreover, climate change may also drive prevailing oceanographic conditions in the spawning habitat of some pelagic fishes out of their optimal environmental window (Guisande et al., 2004). Therefore, it can be projected that the magnitude of the impact of these factors is diverse and depends on the region and the locality of the pelagic species, its biology, and its ecology. In particular, the incidence of global change may be more pronounced in pelagic individuals inhabiting semi-enclosed seas such as the Mediterranean and their sub-basins, which also are characterised by a high pressure of anthropogenic stressors (Coll et al., 2012; Kogovšek et al., 2018). This could be due to the fact that the Mediterranean is an oligotrophic sea, only accounting for 1% of global primary productivity (Uitz et al., 2010), and so any factor that

reduces or hinders access to these resources could pose hazards for pelagic fish. Likewise, pelagic species that are not largely tolerant to increases in temperature, such as sardines, find difficult to migrate northwards or to other areas with lower temperatures in a basin with a limited dispersal potential due to a degree of enclosure of over 99% (Ben Rais Lasram et al., 2010; Lejeusne et al., 2010; Cheung et al., 2013).

After considering the above, it is understood that the confluence of different factors, among which environmental variables are decisive, ends up determining the organism's response in terms of somatic condition, energy storage and health status, and, ultimately, the translation of these factors into reproductive dynamics and potential. Besides, it is important to analyse the state of health of the European sardine in highly exploited areas, as well as areas in which the incidence of environmental change may be pronounced, and to monitor these stocks over time. In these terms, the main objectives of this work have been: 1) to analyse the role of different lipid reserves in the capital breeder European sardine in the northernmost section of the most septentrional, semi-enclosed basin of the Mediterranean Sea (the North Adriatic Sea, specifically, in the Gulf of Trieste, relevant in the Italian capture of sardines), and 2) to study the effect of some environmental variables on the physiological and reproductive status of this fish stock.

2.2. Materials and methods

2.2.1. Sampling collection

Specimens of the European sardine, *Sardina pilchardus*, (N = 704) were collected seasonally from 2019 to 2021 along the Gulf of Trieste (coordinates: 45°40′ N 13°35′ E; surface area: 550 km2; average depth: 18.7 m; water volume: 9500 km3) in the North Adriatic coast (Mediterranean Sea, GFCM—GSA 17) by commercial fisheries (Figure 1). Immediately after the purchase, samples were frozen at −20 °C, which has been demonstrated to have no effect on the explained variables (Brosset et al., 2015a). All specimens in this study were the minimum landing size for a sardine (total length (L_T) of ≥ 11 cm) in the Mediterranean Sea, including the Adriatic (Zorica et al., 2016; Popescu, 2018).

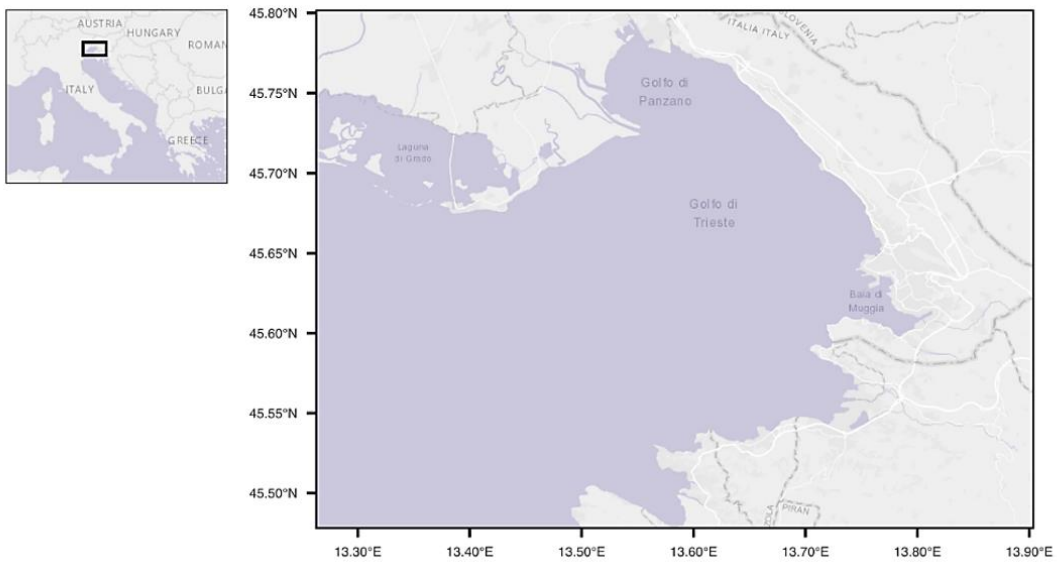

Figure 1. Map of the European sardine sampling area (Gulf of Trieste) and its situation in the Mediterranean Coast.

2.2.2. Somatic condition evaluation

Each sardine individual was measured (total length, L_T, ± 0.1 cm) and weighed (total body weight, W_T, ± 0.01 g; eviscerated body weight, W_E, ± 0.01 g). Gonads (W_G) (± 0.0001 g) and livers (W_L) (± 0.0001 g) were also weighed. A proxy for general somatic condition (Albo-Puigserver et al., 2020) was obtained by the calculation of the relative condition index (Kn) (Le Cren, 1951), interpreted as a higher-than-average physical condition for an individual when Kn exceeds 1, and lower condition when it does not reach this figure, as follows:

$$Kn = \frac{W_E}{W_r} = \frac{W_E}{\alpha {L_T}^{\beta}}$$

where W_E is the eviscerated body weight of an individual, W_r corresponds to the predicted eviscerated weight of an individual of a given total length, L_T is the total length, and α and β are coefficients obtained by the regression line of the logarithms of length and mass (α = 0.056101092, β = 2.1984). The gonadosomatic index ($GSI = 100 \cdot \frac{W_G}{W_E}$) was calculated as an indirect method to estimate the energy destined to reproduction or reproductive effort (Somarakis et al., 2004; Brosset et al., 2016). The hepatosomatic index ($HSI = 100 \cdot \frac{W_L}{W_E}$) was also obtained.

Regarding the lipidic body condition, tissue fat content (i.e., muscle total lipids) was estimated by the average of both sides along the lateral line of each individual using a fish

fat meter (Distell Model FM 992) (Kent, 1990) calibrated for the European sardine. Furthermore, a visual scale for fat mesenteric reserves (Van Der Lingen & Hutchings, 2005) was applied. The vacuity index ($\%\ Vi = 100 \cdot \frac{E}{N}$) was calculated as the number of empty stomachs (E) divided by the total number of stomachs analysed (N).

2.2.3. Reproduction analysis

The sex of each specimen was visually and macroscopically determined, and gonads were classified according to the criteria of Brown-Peterson et al. (2011) into the following categories: immature (they have not reached sexual maturity); developing (gonads increasing in size with gametes that are beginning to develop); spawning-capable (ready for reproduction, but has not begun to spawn); actively spawning (expelling gametes); regressing (gonads almost empty of gametes); and regenerating (mature but reproductively inactive).

2.2.4. Statistical analysis

Analyses were carried out making use of R software version 3.5.1. (R Development Core Team, 2018). Differences among categories were considered as statistically significant if p-value < 0.05. Significance values were indicated as follows in the Results section: p-value < 0.05*; p-value < 0.001**; p-value < 0.0001***. When continuous dependent variables were involved, the Shapiro–Wilk test was applied to test the assumption of normality and Levene's test was executed to prove the homogeneity of variances (Zar, 1996) in all parameters. If both assumptions were met, an independent two-sample *t*-test, a one-way analysis of variance (ANOVA), or Multi-factor Analysis of Variance tests, when corresponded, were performed. Conversely, if both assumptions of normality and equality of variances were not met, the data was transformed to normality. When only a homoscedasticity assumption was violated, data was analysed with Welch's *t*-test. For those parameters in which normal distribution was lacking but homoscedasticity was present, the Kruskal–Wallis analysis of variance was applied. When required, multiple comparison or post hoc tests (Tukey's range test, Dunn's method with Bonferroni adjustment, or Games–Howell test, when corresponded) were applied to the identified different categories. When ordinal dependent variables were involved, the Wilcoxon rank sum test with continuity correction was used. For qualitative dependent variables, Pearson's chi-squared test was performed.

The Spearman's rank non-parametric correlation test between pairs of variables was used to explore the relationships between Kn, GSI, HSI, and tissue and mesenteric fat content, along with environmental variables such as sea surface temperatures (SST; °C) (NOAA High Resolution SST data), chlorophyll-a concentrations (Chl; $mg \cdot m^{-3}$) (NASA combined-satellite), and ocean productivity available to fish (OPFish; %) values, which is an index that characterizes 10–20% of the global phytoplankton production that effectively fuels higher trophic levels (Druon et al., 2021) (Environmental Marine Information System (2022, April 4)).

2.3. Results

2.3.1. Somatic and reproductive condition analyses. Correlation with environmental parameters

The total body length (L_T) of the sardine specimens varied from 10.70 to 16.00 cm (mean ± SD: 13.37 ± 0.87 cm), while total body weight (W_T) ranged from 11.40 to 35.60 g (19.18 ± 3.76 g), with larger and heavier female sardines compared to males (p-value *** for both length and weight) (Table 1).

Table 1. Summary of the variables/indices comparing males and females of the European sardine (*S. pilchardus*) in the Gulf of Trieste. L_T: total length (cm); W_T: total weight (g); W_E: eviscerated weight (g); Kn: relative condition index; GSI: gonadosomatic index (%); HSI: hepatosomatic index (%); % Vi: stomach vacuity index (%). Significance values: p-value < 0.05*; p-value < 0.0001***; NS (not statistically significant).

Variable/ Index	Mean ± SD		Outcome	N	Test	Statistic	P-value
	Males	Females					
L_T (cm)	13.17 ± 0.80	13.50 ± 0.90	Males < Females	704	One-way analysis of means (not equal variances)	F = 27.04	***
W_T (g)	18.07 ± 3.40	19.98 ± 3.80	Males < Females	704	Welch two sample t-test	t = 7.00	***
W_E (g)	16.01 ± 2.84	17.64 ± 3.23	Males < Females	704	Welch two sample t-test	t = 7.11	***
Kn	0.982 ± 0.113	1.023 ± 0.112	Males < Females	704	Welch two sample t-test	t = 4.78	***
GSI	1.906 ± 2.013	2.547 ± 2.268	Males < Females	704	One-way analysis of means (not equal variances)	F = 15.54	***
HSI	0.690 ± 0.447	0.785 ± 0.491	Males < Females	704	Welch two sample t-test	t = 2.62	*
Tissue fat content	9.468 ± 4.333	10.353 ± 4.469	Males < Females	704	Welch two sample t-test	t = 2.63	*

Mesenteric fat	-	-	Males = Females	704	Wilcoxon rank sum test with continuity correction	W = 60366	NS
% Vi	-	-	Males = Females	704	Pearson's chi-squared test	$\chi^2 = 1.31$	NS

Further significant general differences between the sexes were obtained for tissue fat content (p-value *), Kn (p-value ***), GSI (p-value ***), and HSI (p-value *) (Table 1), with higher values for females in the parameters mentioned (Figure 2B,D–F). No general differences were recorded for mesenteric fat content between sexes (p-value = 0.891, NS) (Figure 2C). When reproductive developmental stage was included, significant differences in tissue fat content between the sexes only were reported for the spawning-capable stage (p-value *), which were higher in females. A significant interaction has been observed in HSI combining developmental stage and sex (p-value *) (Figure 3B). For GSI, significant differences have been seen among developmental stages for this index (p-value ***), with sex differences only at the actively spawning stage (p-value ***) (Figure 3A). Kn did not significantly vary with developmental stage, but it did with month (p-value ***), and had the lowest values registered from December 2019 until May 2020 in both sexes, following an increasing trend towards 2021.

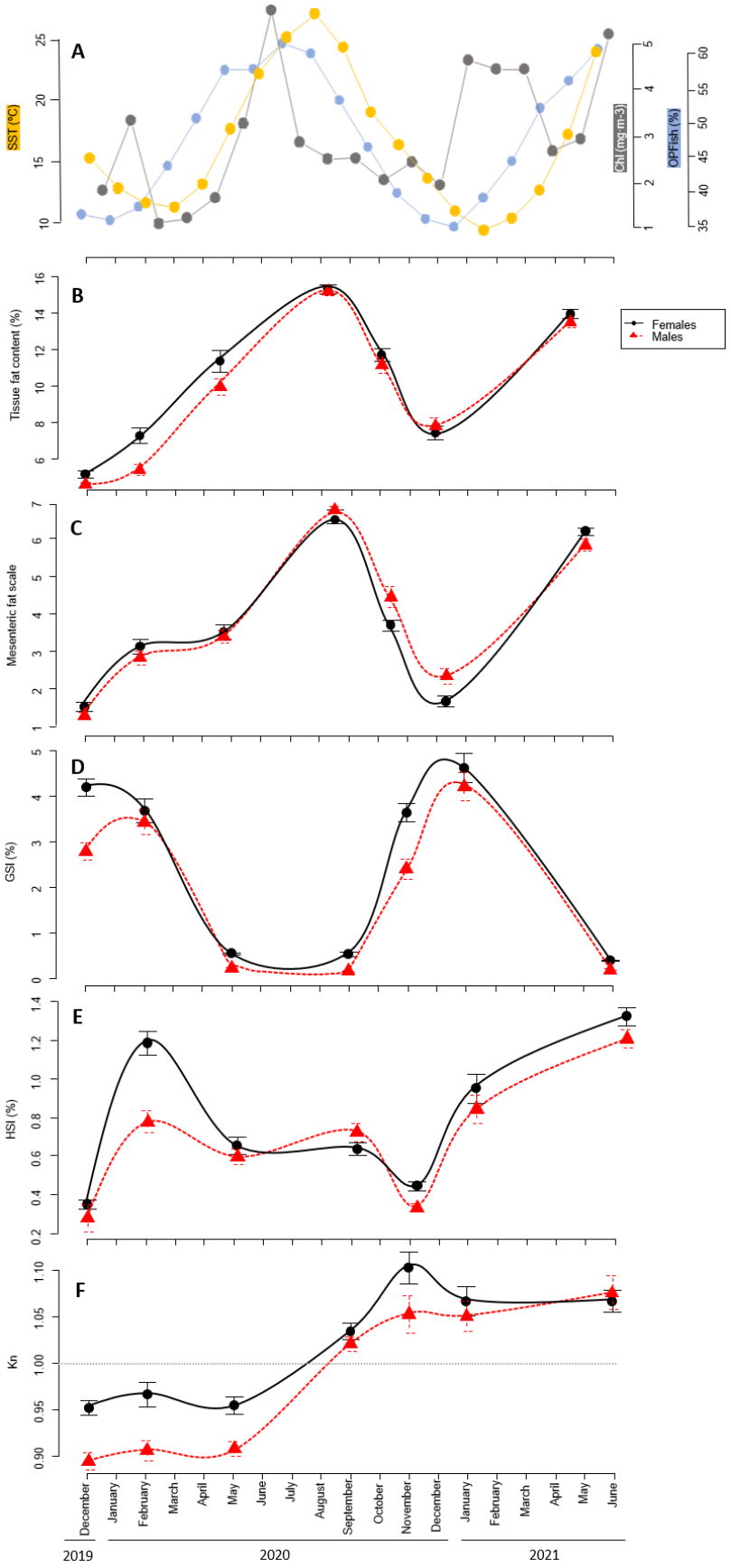

Figure 2. Condition analysis parameters in the European sardine (*S. pilchardus*) and the averages of environmental variables along the Gulf of Trieste (North Adriatic, Mediterranean Sea). **A.** Monthly mean sea surface temperature (SST (°C); yellow line),

chlorophyll-a concentration (Chl (mg·m^{-3}); grey line), and available portion of productivity for the fish (OPFish (%); blue line) in the study area over the sampling time (2019–2021). **B**. Tissue fat content (%), **C**. Mesenteric fat scale, **D**. Gonadosomatic index (GSI; %), **E**. Hepatosomatic index (HSI; %), and **F**. Relative condition factor (Kn) were estimated by sex (females, black line and dots; males, red line and triangles) as averages of the individual measurements.

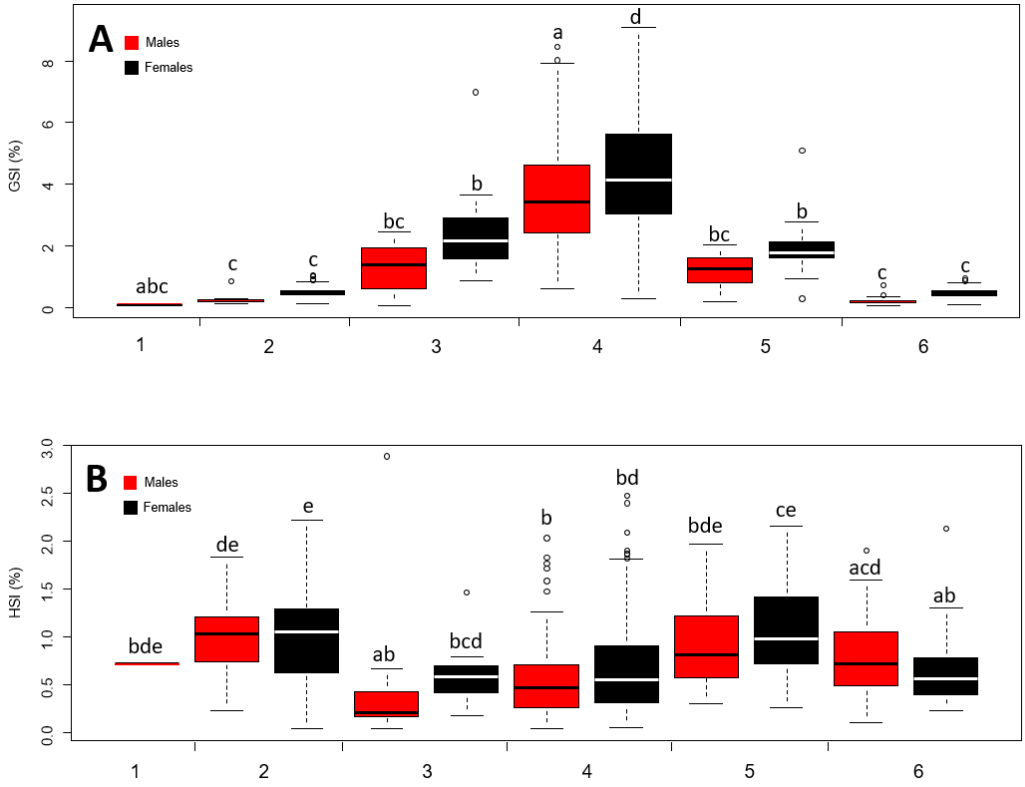

Figure 3. Gonadosomatic and hepatosomatic indices by sex and reproductive developmental stage in the European sardine (*S. pilchardus*). **A**. Gonadosomatic index (GSI) by sex and reproductive developmental stage. **B**. Hepatosomatic index (HSI) by sex and reproductive developmental stage according to the classification of Brown-Peterson et al. (2011). Different letters on the graph indicate significant differences among stages and/or between sexes. Outliers are marked with a circle (°).

Furthermore, Kn and GSI were not significantly related, even though Kn was positively correlated with both tissue and mesenteric fat content and was more accused with the former in both sexes (Figure 4). Kn was also positively linked to chlorophyll-a in both sexes and was more pronounced in the case of males (ρ = 0.46***), while it was lightly related with SST. No significant relationship was observed between Kn and OPFish.

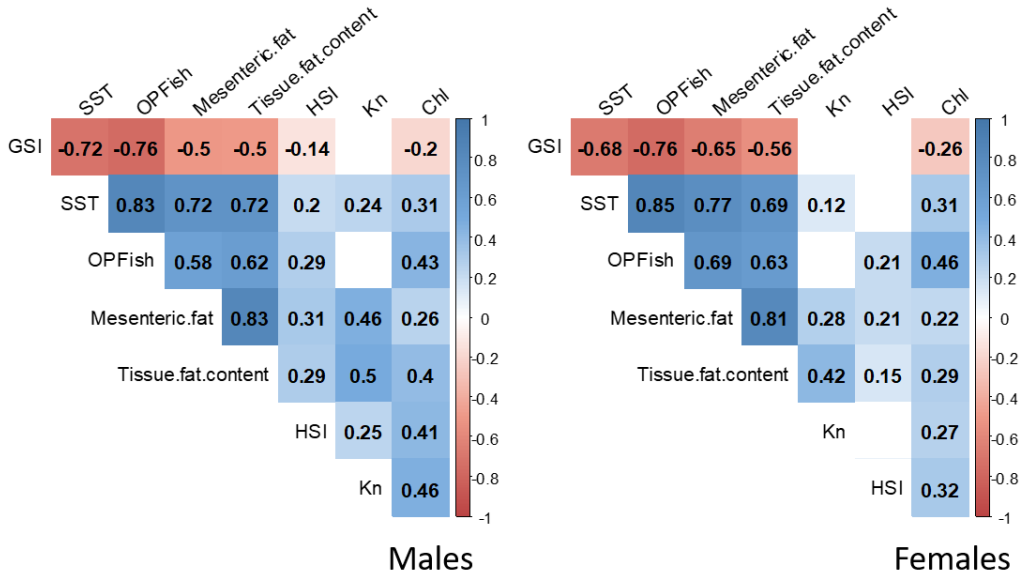

Figure 4. Spearman correlation matrix among the European sardine's (*S. pilchardus*) condition parameters and the environmental variables considered in males (**A**) and females (**B**). Relative condition index (Kn), tissue fat content (%), mesenteric fat scale, gonadosomatic index (GSI; %), and hepatosomatic index (HSI; %) were correlated with each other and with the following environmental variables: sea surface temperature (SST; °C), chlorophyll-a concentration (Chl; mg·m^{-3}), and available portion of productivity for the fish (OPFish; %). The colour gradient from maroon to navy blue corresponds to the correlation with strength, from negative to positive, respectively. The empty squares represent a non-significant correlation according to a p-value of < 0.05*.

Seasonal trends can be inferred from data, with opposite patterns between GSI and tissue and mesenteric fat content for both sexes (Figure 2), as was confirmed by the Spearman's correlation test (ρ = −0.5 for males and −0.56 for females, for tissue fat content; and ρ = −0.5 and −0.65, respectively, for mesenteric fat) (Figure 4). While GSI decreased after winter in both 2020 and 2021, lipid content values started to increase. Tissue fat content values increased in a progressive way from winter, while a steeper slope linked to a later accumulation of mesenteric fat was observed in the late spring of 2020. Moreover, correlations indicated that mesenteric and tissue fat content were strongly related for both sexes (ρ = 0.830*** for males and ρ = 0.813*** for females). Chlorophyll-a was positively correlated with tissue fat content and more than with mesenteric fat in both sexes. However, a correlation close to −0.2 was seen between chlorophyll-a and GSI. Further, SST was strongly and positively related to tissue and mesenteric fat in a similar way in females and males, and a strong negative correlation when related to GSI (ρ = −0.72*** for males, ρ = −0.68*** for females) was observed. Values of productivity available to fish (OPFish) were over ρ = 0.58 for both tissue and mesenteric fat in both sexes, although higher in the case of

females, especially regarding mesenteric fat (ρ = 0.69***). At the same time, OPFish was highly negatively correlated to GSI (ρ = −0.76***) for both sexes. HSI figures were disparate, although the lowest values of the cycle were detected in the winter months for both sexes, and they were positively related with chlorophyll-a and only slightly with SST in the case of males and with OPFish, showing a correlation over ρ = 0.21.

Vacuity index values (% Vi) by season were 5.56% in Autumn 2019, 46.08% in Winter 2020, 47% in Spring 2020, 11.46% in Summer 2020, 9% in Autumn 2020, 0% in Winter 2021, and 29% in Spring 2021.

2.3.2. Sex ratio and reproductive cycle

The overall sex ratio (m/f = 0.710) deviated significantly from the hypothetical distribution of 1:1 (χ^2 = 20.144, *df* = 1, p-value ***), as more females were observed than males. However, significant differences among sex proportions were not seen monthly.

The early beginning of the reproductive season was recorded in Summer (September 2020) for only one specimen, a female individual. However, the greater percentage of active spawners were observed in the autumn and winter months for both sexes during the different sampled years. Thus, the spawning period in the Gulf of Trieste lasted at least from September–October to March.

During the sampled months of the spring and summer, 2020, and the spring of 2021, a higher percentage of developing individuals corresponded to females, a sign of their more advanced gonad maturation than males over the years, in which a large proportion of them were at the regenerating phase. However, the values during the reproductive seasons were again similar in both sexes (Figure 5).

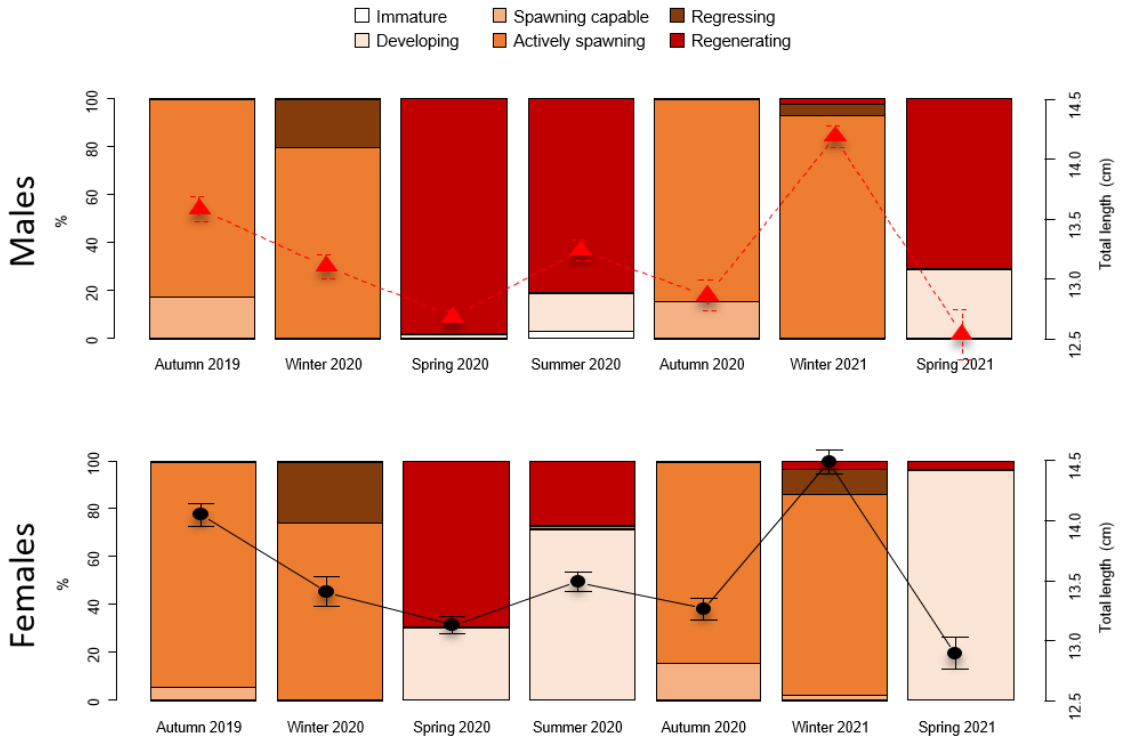

Figure 5. Seasonal analysis of the European sardine's (*S. pilchardus*) reproductive developmental stages in the Gulf of Trieste (North Adriatic). Graphs are presented by sex and reproductive developmental stage according to the classification of Brown-Peterson et al. (2011). The total length (cm) is also reflected for both sexes.

2.4. Discussion

Several studies suggest the significant effect of spawners' condition on reproductive potential (e.g., Marteinsdottir & Begg, 2002; Wright & Trippel, 2009; Alonso-Fernández, 2011), especially when it comes to the small pelagic species among which the sardine is found (Rosa et al., 2010; Albo-Puigserver et al., 2017; Saraux et al., 2019; Albo-Puigserver et al., 2020). Therefore, it was considered important not only to estimate the reproductive period seasonality and/or the investment in reproduction (i.e., GSI), but also to assess the lipid storage, somatic condition, and health status of the fishing resource, the European sardine, in the study area.

The Northern Adriatic Sea system is one of the major chlorophyll hot spots in the Mediterranean Sea, and it has been recognised to depend on the water and nutrient discharge from the Po River and a dozen small rivers that flow into the Adriatic Sea north of the Po River delta and in the Gulf of Trieste (i.e., the Isonzo River (Conversi et al., 2009)) (Viaroli et al., 2015), with around 40% of the chlorophyll production of the whole Adriatic (Rizzi et al., 2016). These fresh water sources have a major impact on phytoplankton

biomass due to the nutrients loads and to local upwelling events and eddies that contribute to spread the discharges offshore and enhance primary production (Brosset et al., 2017). In fact, it has been reported that the Northern and Central Adriatic are dominated by the pelagic compartment, with especial reference to plankton and small pelagic fish, among which the anchovy and sardine stand out (Morello & Arneri, 2009). Evidence of this influence of riverine inputs on the productivity of small pelagic fish has been provided in several studies (Lloret et al., 2004; Vargas-Yáñez et al., 2020; Caballero-Huertas et al., 2022c), reinforced by the fact that sardine catches from inshore waters are generally in better condition than those from offshore waters (Mustać & Sinovčić, 2010). However, there is variability linked to seasonality, as zooplankton abundance and feeding opportunities differ due to changes throughout the year. For example, in spring, the optimum feeding condition is inshore, while at the end of summer, the offshore offers larger amounts of zooplankton, which contributes to triggering migratory behaviour in sardines (Morello & Arneri, 2009).

Direct energy flow from planktonic filter-feeding goes to gonadal development and egg production, implying that in addition to capitalized energy, sardines also use current income for supporting reproduction (Ganias, 2009). In fact, before the spawning period, storage lipids, as well as other nutritional compounds (such as proteins, vitamins, and minerals) in muscles, the liver, and visceral organs, are mobilized to the gonads to ensure maturation (Pacetti et al., 2013). This coincides with the opposite patterns observed in our data between tissue fat and/or mesenteric fat content and GSI curve, which was also confirmed by correlation analyses. These opposite trends among fat reserves and gonad growth have been previously documented in various studies (Ganias et al., 2007; Mustać & Sinovčić, 2009; Albo-Puigserver et al., 2020). Nevertheless, the relative condition results obtained by Kn did not show an apparent relationship with GSI, contradicting our projections, as well as our previous observations (Ganias et al., 2007). However, they were in line with Campanini et al. (2021), which suggests that Kn cannot be considered a good proxy for the energy density of sardines, while fat meter analysis appears to be a suitable method to evaluate the energy content of this species. Further, other authors have suggested that Kn data should be carefully analysed because it is population-/stock-specific (Lloret et al., 2013). In addition, we can comment that Kn varied from the reproductive season of 2019–2020 to 2020–2021, and we observed Kn average values under 1 in the former and over this figure in the latter winter period, with an increasing trend from the beginning of the sampling. These differences did not seem to be reflected in terms of GSI from one cycle to the other, although in this case, they coincided with more favourable

conditions (i.e., a higher average concentration of chlorophyll-a in the area and lower average SST) in winter 2021 than that recorded in winter 2020.

Moreover, according to Ganias et al. (2007), the seasonality of spawning did not match the variations of HSI during the sampling period, and no relationship was proven between HSI and GSI, as was also reflected by Somarakis et al. (2004). GSI values in our study were similar to those reported by Mustać and Sinovčić (2010) in the Adriatic, since they were the highest when the lowest annual values of sea temperature were recorded. However, despite being correlated, GSI and chlorophyll-a showed a slight negative ρ correlation coefficient, while the proportion of productivity available to fish (OPFish) showed a greater correlation with GSI and with tissue and mesenteric fat content. This suggests that chlorophyll-a and fish were not directly related, but a higher chlorophyll-a concentration might be an indicator of favourable conditions for sardines (Bellido et al., 2008; Fernández-Corredor et al., 2021). This could be occurring because, as we should not forget, OPFish values are related to the concentration of chlorophyll-a since they are data derived from chlorophyll-a horizontal gradients (Druon et al., 2021), taking into account that which would be usable for a species such as the sardine.

In the present study, Figure 2A shows that at the times when amounts of chlorophyll-a are not very large, the proportion of available resources is quite high, occurring in the months immediately prior to sardine spawning, and decisive for performance during the reproductive time. Moreover, it maintained a more stable and cyclical trend than general chlorophyll-a values throughout the sampling years. Thus, the amount of environmental chlorophyll-a seems to be reflected in Kn, although it does not necessarily translate into the GSI. Thus, the direct available resources (OPFish) (see Figure S2.1 as an example in the study area) seem to better illustrate the direct implication that resources have in reproductive terms, as well as SST, in line with Druon et al. (2021), confirming that in order to project sardine production, the impact of abiotic factors (i.e., temperature) on reproduction should be taken into account, as they seasonally affect their distribution. The prevalence of oocyte atresia together with spawning incidence seem to be positively affected by water temperature, whilst the index of zooplankton production significantly correlates with relative fecundity (Ganias, 2009). In addition to the fact that temperature and food availability can shape the intensity of reproduction and quality of the eggs, thereby affecting reproductive potential, they could also condition the survival of the larvae (Garrido et al., 2017). The amount of yolk in an egg affects the time that larvae can survive without food, and so the effect of temperature on absorption rate should also be considered when relating larval survival to egg quality (Riveiro et al., 2000). Therefore, special attention should be

paid to the Gulf of Trieste, where sea surface temperature has increased 0.5 °C over a period of almost four decades (Conversi et al., 2009), and 0.36% year^{-1} in the Northern Adriatic (Grilli et al., 2020).

Garrido et al. (2008b) commented that the muscle tissue of male and female sardines engaged in spawning showed no significant differences in total fatty acids concentration in sardines from Portugal (Atlantic Ocean). In this regard, our analysis showed that in general, females contained a higher muscle fat content, which was especially reflected during the spawning-capable phase, and during the actively spawning season, these differences were not significant. In this way, significant linear relations have been found between fatty acid concentrations in female sardine muscle and oocytes (Garrido et al., 2007b). Moreover, significant differences between the sexes in GSI were identified only for active spawners (Figure 3A), with larger values observed in females. Our results are similar to those of Basilone et al. (2021) for a study in the Central Mediterranean, although they differed from other studies in which males were identified as the individuals with the highest values of GSI (Nunes et al., 2011). According to Ganias et al. (2014), isometric ovarian growth has been shown for all the developmental stages in sardines except for hydration, and so the relative weight of the ovaries (i.e., the gonadosomatic index, GSI) remains stable with body size, except in this phase (i.e., corresponding to hydration and ovulation in females (Brown-Peterson et al., 2011)). A similar effect has not been previously analysed for gonad developmental phases in males (Nunes et al., 2011).

Mesenteric lipid content and the total fat content showed similar trends and large positive correlations (ρ = 0.81–0.83), as supported by Mustać and Sinovčić (2009) in the eastern Middle Adriatic, which could suggest that the accumulation of fat around viscera, within the muscle, and between skin and muscle takes place in parallel (Nunes et al., 2011). However, although no significant differences were seen between the sexes, we observed a slightly stronger negative correlation between mesenteric fat content and GSI in females than in males, and higher than the relationship among tissue fat content and GSI. While in males, ρ was equal to −0.5 for both variables, in females we detected a ρ of −0.56 for tissue fat content and a ρ of −0.65 for mesenteric fat. This result is expected because, as we have observed, the investment of females in the reproductive season in gonad growth is greater than that of males (Figure 2D and 3A), being more pronounced in the fall of lipids in these months (Figure 2B, C), and especially regarding mesenteric fat. Most of the energy destined to reproduction is accumulated in the viscera or mesentery (Abdelmoulah & Hadj Ali Salem, 1981). While muscle is a more stable fat store, mesenteric fat is much more labile and likely to be the first fat store to become depleted during gonad maturation, as well as the first fat

store to respond to increased food intake (McPherson et al., 2011). In fact, Krzeptowski (1983) suggest that fat in viscera could reflect a much more intensive turnover of body- and energy-producing matter in females during spawning and after it, at the time of recovery of spent gonads. Therefore, the relationship between GSI and fat in females may be higher and even more pronounced when it comes to the parameter of mesenteric fat. Given these results, our analysis contradicts the results of Somarakis et al. (2004), who reported that fat storage stage does not substantially affect GSI. Although chlorophyll-a concentrations in the environment are more strongly related with tissue fat content than with mesenteric, available resources are similarly correlated with both types of fat reserves for both sexes. Observing Figure 2B, C, there was a monthly progressive increase after the reproductive period in tissue fat content, while mesenteric reserves increased rapidly in a short period of time, coinciding with the moment in which the vacuity index dropped from the spring to the beginning of summer to be mobilised rapidly in the development of the gonads. An interesting aspect to highlight is the different vacuity index found in the winter of 2021 (0 %) with respect to that registered in the winter of 2020 (46.08 %), although high inter-annual variability in feeding intensity has been previously recorded (Garrido et al., 2008a). In fact, the recorded differences may be related to the fishing time, as it has been documented that during summer, *S. pilchardus* feed continually during daytime with a peak at around sunset, while during winter, high feeding rates occurred only in the early night (Nikolioudakis et al., 2011), and so it is likely that the catch occurred right after the time of the food intake.

Moreover, sex-biased sex ratios towards females could be highlighted in our results, which were also observed by Zorica et al. (2017) in the Eastern Adriatic Sea. The authors documented sardine spawning activity from the beginning of October until the end of April, with a main peak between November and February, over recent decades in the Adriatic Sea (Zorica et al., 2020). However, spawning sardine stock in the Northern Adriatic has been established by Nejedli et al. (2004) from August–September to May. According to our data, in the Gulf of Trieste, we started to identify the bulk of active spawners before November, accompanied by a significant increase in GSI values which continued to grow at least until February. No active spawners were identified in subsequent sampled months. In May, only individuals in a state of regeneration and early development could be observed. In this way, we can deduce that our results do not differ in a global way from those in the Adriatic proposed by Zorica et al. (2020). Differences by sex in the percentage of developing gonads were observed in our study during spring (Figure 5), with more developing females compared to male individuals over the pre-spawning seasons. In spring in the North Atlantic Moroccan area, a higher percentage of females in a more advanced gonadal stage was also

reported, even though the percentages of the different stages were equal in the reproductive season (autumn–winter) (Abderrazik et al., 2016), as shown by our data. It is probably related to size, as Ganias et al. (2007) reported that a smaller size may contribute to delayed gonadal maturation, coinciding with the significantly lower sizes for males in our study. Further, the faster recorded recovery of females compared to that of males regarding energetic indices, even after a previous greater investment, could be translated into a higher capacity of the gonadal development and oocyte maturation process.

2.5. Conclusions

In this study, we intended to shed light on the sardine's somatic condition due to its essential role in reproductive potential and performance within an environmental framework in a highly productive Mediterranean area, which was especially vulnerable to impacts (i.e., fishing pressure and high degree of enclosure, among other factors). Direct lipid measurement indices (tissue fat content and mesenteric fat scale) seemed to be suitable to be used to project their contribution to sardine reproduction after we found differences linked to sex, as occurred with the GSI parameter for active spawners, which was higher in females. In addition, reproductive cycle by sex showed a more advanced gonad maturation in females, although this became similar during the active reproductive season. Current and future threats to the stock status of sardines should foster to continuously monitor those determining indicators of health status and reproductive potential to manage the resource effectively. It is suggested to include environmental variables (i.e., SST, chlorophyll-a, and OPFish) in condition and reproductive studies, and to analyse their implications in sardine stocks over time.

Chapter 3

Unravelling the drivers of variability in body condition and reproduction of the European sardine along the Atlantic-Mediterranean transition

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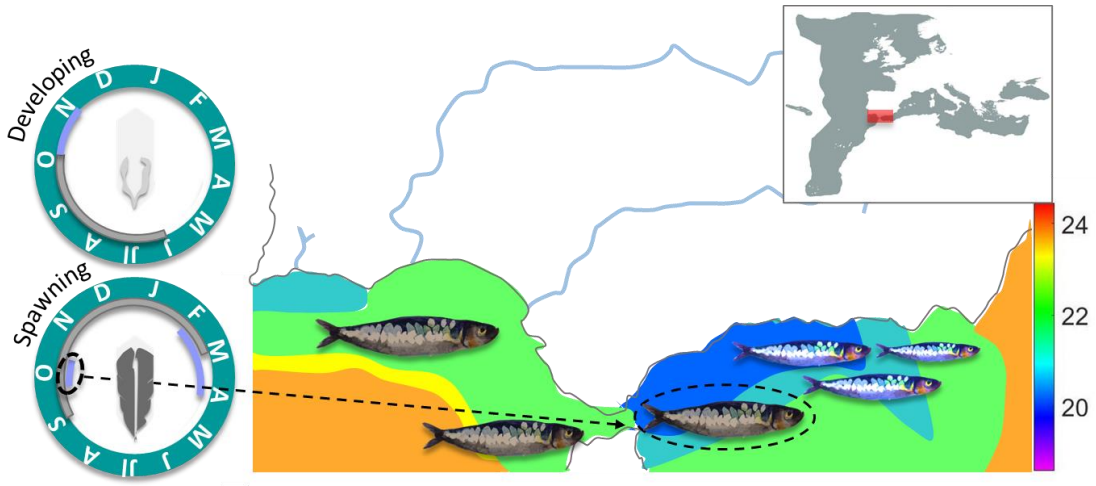

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Abstract

Body condition and reproduction data are broadly used to assess the health status of fish because of its implications for recruitment and ecosystem structure. *Sardina pilchardus* is a small pelagic distributed throughout both Mediterranean and Eastern Atlantic. Seasonal trend analysis of energy storage and reproduction was carried out in sardines from two areas along the Atlantic-Mediterranean transition: Southern Portugal-Gulf of Cádiz (POR-GC) (Atlantic Ocean) and Alboran Sea (Alb) (Mediterranean Sea) from 2019 to 2021. Energetic condition was estimated using tissue and mesenteric fat content, hepatosomatic index (HSI), and the relative condition factor (Kn). Sex, reproductive developmental stage, and gonadosomatic index (GSI) were also obtained. In addition, the oceanographic and meteorological characteristics of the areas were analysed. Results showed that seasonal Kn, tissue and mesenteric fat content, and HSI values of POR-GC specimens exceeded Alb's with summer arrival, period in which sardine acquires reserves to allocate them to reproduction. These differences could be associated to greater productivity of the former area mainly due to rivers discharges and trade winds intensification during summer (from July to September). Furthermore, gonad maturation of POR-GC stock occurred before the Alb. However, no spawning capable individuals were identified until February in POR-GC. In contrast, in Alb it was observed a remarkable fraction of spawning capable and active spawner individuals in October. We hypothesized the migration of mature individuals from POR-GC to the spawning areas located in the Alb. Seasonal genetic population studies are required to untangle it and reliably evaluate the environmental effect on the stocks.

Keywords: Alboran Sea • Gulf of Cádiz • Lipid content • Oceanographic variables • *Sardina pilchardus* • Spawning migration

3.1. Introduction

The small pelagic clupeid European sardine (*Sardina pilchardus* (Walbaum, 1792)) is distributed along the Eastern Atlantic, from the North Sea to the Mauritania/Senegalese coasts, spanning from the Azores to the Mediterranean and the Black Seas, with the biggest populations and fisheries concentrated in the Atlantic coasts of north Africa and Europe (Stratoudakis et al., 2007). As forage species that feeds primarily on plankton, it plays an important ecological role in the ecosystem mainly due to its contribution to higher trophic levels at bottom-up scale (Cury et al., 2000). As one of the most commercialized species in areas such Southern Europe, lower values in both number of individuals and biomass have been pointed out, consequence of the high demand for sardines and consecutive downward trend in terms of stock abundance in the last years, added to other environmental pressures (Baptista et al., 2019; FAO, 2018, 2020). European sardine is a winter batch spawner with indeterminate fecundity, which accumulates lipids from spring to autumn to allocate them to somatic growth and reproduction, reproductive strategy that has been defined as capital breeding (Ganias, 2009; McBride et al., 2015). Thus, sardine's spawning mainly occurs during the coldest months of the year in both Atlantic and Mediterranean basins (Bandarra et al., 2018; Ganias, 2009; Stratoudakis et al., 2007). However, both batch fecundity and spawning frequency can vary considerably with areas, within the spawning season, between years, and with age and size, since sardine and other clupeoids display reproductive plasticity, being able to change their reproductive traits whenever the environmental conditions require or allow it (Ganias, 2009). Thus, variability in body condition and energy storage has important implications for recruitment (Albo-Puigserver et al., 2020), and morphometric indices and analysis of lipid content/bioenergetic index seem to be good complementary indicators for condition indices in sardine (Brosset et al., 2015a). Therefore, they result of great value for the study or inference of the quality of spawning and recruitment (Van Der Lingen & Hutchings, 2005).

It is a fast-growing species with a relatively short life span and early maturation which, in addition to its significant interannual fluctuations in population recruitment and biomass, make sardine stocks especially difficult to manage (Blaxter & Hunter, 1982; Nunes et al., 2011) to ensure a sustainable fishery. Moreover, in recent decades, important changes in abundance, landings and biological features have been reported for this species, partially attributed to increases in fishing pressure and environmental fluctuations (i.e., variations in oceanographic parameters) (Albo-Puigserver et al., 2020; Van Beveren et al., 2014; Vargas-Yáñez et al., 2020). The impact of environmental variables such as temperature, salinity, and chlorophyll concentration on sardine habitat suitability has been notably

reported in a significant number of studies (e.g., Jghab et al., 2019; Lloret-Lloret et al., 2022; Vargas-Yáñez et al., 2020). This is because environmental fluctuations can affect fish populations controlling food quantity and/or quality, directly influencing growth, condition and annual recruitment (Brosset et al., 2015b), being early life stages particularly sensitive (Fernández-Corredor et al., 2021). Moreover, as European sardine is close to the bottom of the pelagic food web and feed directly on phytoplankton and micro- and mesozooplankton prey, it is likely to be rapidly affected by spatial and temporal variability of the producers and first order consumers (Garrido et al., 2008b).

Thus, comparative studies between stocks at species level are very important since they elucidate the effect of habitat conditions on growth and recruitment of fishes (Basilone et al., 2017). Environmental complexity makes investigating the effects of climate variability along fish distribution particularly challenging (Tsikliras et al., 2019). Besides, genetic population structure and connectivity in the marine environment, especially, when it comes to pelagic species, make this task even more difficult (Caballero-Huertas et al., 2022a).

The Southern Iberian coastal systems consist of two adjacent basins, the Southern Portugal-Gulf of Cádiz (POR-GC) and the Alboran Sea (Alb) (connected by the narrow Strait of Gibraltar), with different hydrographic, oceanographic and meteorological characteristics (Figure 1). POR-GC is a warm basin during spring and summer (i.e., warm shelf waters mostly during summer, with an average temperature of 21.3 °C) (Huertas et al., 2005; Navarro & Ruiz, 2006; Prieto et al., 2009), especially in the coastal areas (García-Lafuente et al., 2006) where marshes and riverine influence is particularly high. The central area of the Gulf is highly oligotrophic with the presence of a quasi-permanent anticyclonic gyre (Vargas et al., 2003), whereas in specific coastal areas intermittent upwelling processes occur mainly due to wind forcing (Stevenson, 1977). On the other hand, the north-western (NW) Alb is strongly influenced by the circulation through the Strait of Gibraltar, as the incoming Atlantic Jet feeds and maintains the surface structures in the area (Viúdez & Haney, 1997) including a strong frontal area that divides a productive sector of intense upwelling in the coastal zone (García-Martínez et al., 2019; Vargas-Yáñez et al., 2017, 2019; Viúdez et al., 1996) and a more oligotrophic area far offshore. Thus, due to the different environmental characteristics potentially affecting sardine habitat shaping in both areas, we considered of interest the study of physiological parameters and indexes in E. sardine under the oceanographic and atmospheric factors of both locations. In the case of the Alb Sea and along POR-GC, there are very few studies trying to establish and quantify the relationships between the abundance, distribution and condition of European sardine and other small pelagic fishes with environmental factors (Vargas-Yánez et al., 2020), therefore, our study aims to contribute to improving knowledge in this regard.

The main objectives of this study were to seasonally characterise 1) the differences in the energetic body condition and health status, as well as in the reproductive cycle, between European sardines from two nearby areas, the Southern Portugal-Gulf of Cádiz (Atlantic Ocean) and the Alboran Sea (Mediterranean Sea) coasts, connected by the Strait of Gibraltar, and 2) the spatial patterns of environmental variables to identify those that may be influencing the condition and reproduction of sardine stocks in each area through a descriptive and qualitative analysis.

3.2. Materials and methods

3.2.1. Body condition and reproduction analyses

3.2.1.1. Biological sampling and processing

Specimens of *Sardina pilchardus* (N = 1128) were collected seasonally (monthly samples were averaged per season) from 2019 to 2021 along the Southern Portugal-Gulf of Cádiz coast (Northeast Atlantic Ocean, Portuguese Waters - East (FAO fishing area division 27.9.a)) and the coast of Málaga, bathed by the Alboran Sea (Mediterranean Sea, GFCM – GSA 1) by purse seiners (Figure 1 and Table S3.1). Samples were immediately frozen at - 20 ºC, which has been demonstrated to have no significant effect on the studied parameters (Brosset et al., 2015a). Each sardine was measured (total length, L_T ± 0.1 cm) and weighed (total body weight, W_T ± 0.01 g); eviscerated body weight, W_E ± 0.01 g). Gonads (W_G ± 0.0001 g) and liver (W_L ± 0.0001 g) were also weighed. A proxy of body condition was obtained by the calculation of the relative condition index (Kn) (Le Cren, 1951) ($Kn = W_E / \alpha L_T^{\beta}$), where W_E is eviscerated weight, L_T is total length, and α (0.0045) and β (3.1793) are constants obtained by the regression line of the logarithms of length and mass from the samples. It is interpreted as a higher-than-average physical condition for an individual when Kn exceeds 1, and lower condition, when it does not reach this figure. As a measure of energetic reserves and reproductive activity, the hepatosomatic ($HSI = 100 \cdot [W_L / W_E]$) and gonadosomatic ($GSI = 100 \cdot [W_G / W_E]$) indexes, respectively, were also calculated. The sex of each specimen was determined macroscopically, and gonads were classified according to the criteria of Brown-Peterson et al. (2011) for reproductive developmental stages (i.e., immature, developing, spawning capable, actively spawning, regressing, and regenerating, see Figure 2A). Regarding the lipidic and energetic body condition, tissue fat content (i.e., muscle total lipids) was estimated by the average of both sides along the sardine lateral line using a fish

fat meter (Distell Model FM 992 with SARDINE-2 calibration). This device allows the rapid measurement of water content and provides the relative tissue fat content (%) of each individual due to the inverse relationship between water and lipid content (Bayse et al., 2018; Brosset et al., 2015a). Also, a visual scale for sardine ranging from 1 to 7 was applied to quantify the fat mesenteric reserves (Van Der Lingen & Hutchings, 2005), where 1 = fat lines invisible or thin and indistinct; 2 = depth greater than width of one or more fat lines; 3 = pyloric fat line noticeably thicker than the other fat lines, and about one-third the thickness of the pyloric junction; 4 = depth greater than width for all fat lines but no fat lobes present; 5 = all fat lines slightly lobed, but no overlap between lobes; 6 = fat line lobes obvious and show some overlap; and 7 = fat line lobes large, lots of overlap, and fundulus well-covered with fat.

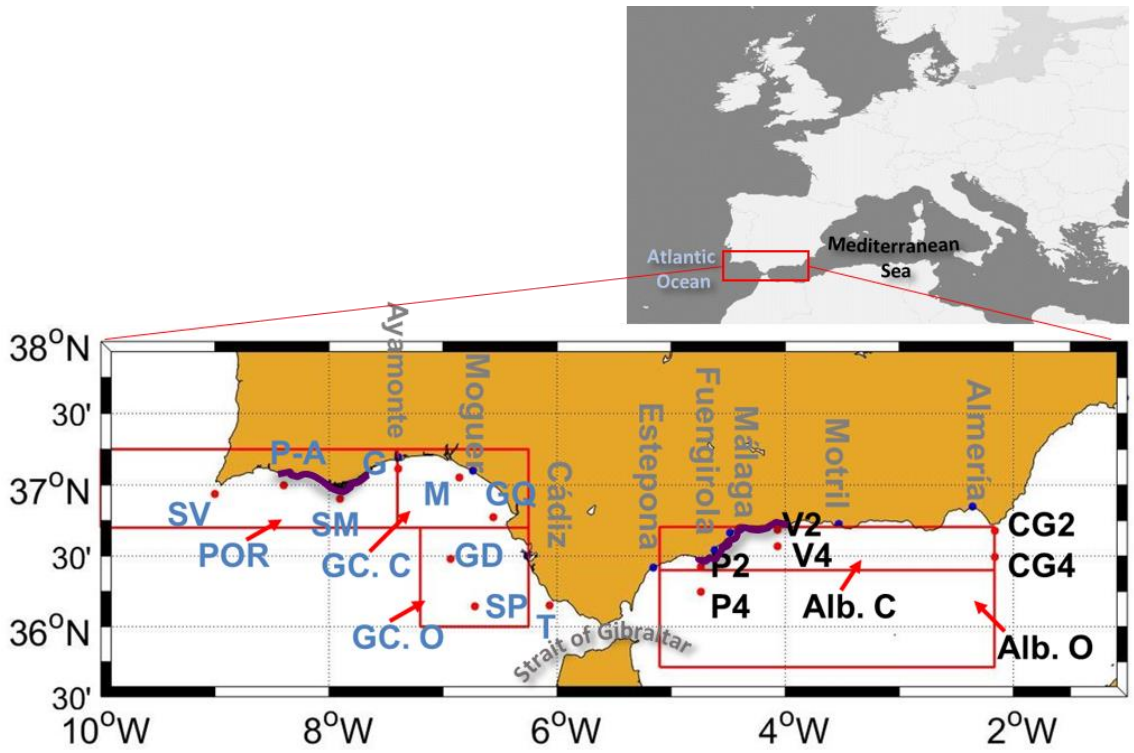

Figure 1. Map of the two defined sample areas. Oceanographic information was obtained from the different stations (red dots) reflected (Southern Portugal-Gulf of Cádiz stations (blue letters) - SV: Cape San Vicente, P-A: Portimao-Albufeira, SM: Cape Santa María, G: Guadiana River mouth, M: Mouth of Rivers Tinto and Odiel, GQ: in front of the Guadalquivir River mouth, GD: Gualdalquivir River offshore, SP: Sancti Petri, T: Trafalgar; Alboran stations (black letters) - P2: Cabopino inshore, P4: Cabopino offshore, V2: Vélez inshore, V4: Vélez offshore, CG2: Cabo de Gata inshore, CG4: Cabo de Gata offshore). Stations were classified according to their location (red rectangles) as follows: POR (Southern Portugal): SV, P-A, SM; GC. C (Gulf of Cádiz, Coastal area): G, M, GQ, T; GC. O (Gulf of Cádiz, Open sea): GD, SP; Alb. C (Alborán Sea, Coastal area): P2, V2, V4, CG2, CG4; or Alb. O (Alborán Sea, Open

Sea): P4. Wind intensity and direction data was collected from the following locations (blue dots): from west to east, Ayamonte, Moguer, Cádiz, Estepona, Fuengirola, Málaga, Motril, and Almería. The purple outline indicates the sardine sampling areas.

3.2.1.2. Statistical analysis

Using R software version 3.5.1. (R Development Core Team, 2018) to examine the differences among areas, seasons, and sex for all the indexes, Shapiro-Wilk test was applied to test the assumption of normality and Levene's test was executed to check the homogeneity of variances (Zar, 1996) in all parameters. When both assumptions were met, independent two-sample t-test (i.e., to compare morphogravimetric parameters between locations), one-way analysis of variance (ANOVA) (i.e., to compare mean values of the biological indexes between sexes in a concrete season or between consecutive seasons for the same sex in an area) or Multi-factor Analysis of Variance (i.e., to examine the effect of area, season, and reproductive stage on the parameters) tests were performed. Pearson's Chi-squared test was applied to compare among categories of qualitative variables (i.e., mesenteric fat by sex). Conversely, if both assumptions of normality and equality of variances were not met, the data was transformed to normality. When only homoscedasticity assumption was violated, data was analysed with Welch's t-test (i.e., GSI by gonadal developmental stage). For those parameters in which normal distribution was lacking but homoscedasticity was present, Kruskal-Wallis analysis of variance was applied (i.e., GSI, Kn, tissue fat content, and HSI by sex). When required, multiple comparison or post-hoc tests (Tukey's range test, Dunn's method with Bonferroni adjustment, or Games-Howell test, when corresponded) were applied to identify the different categories. Statistically significant differences were considered if p-value < 0.05*.

3.2.2. Oceanographic and meteorological analysis

3.2.2.1. Data and methods

A large data set of environmental variables was analysed to characterise those factors that may have some influence on the reproductive cycle and general health/condition of European sardines. We collected data with different spatial resolution and different geographical extension to have information on those meteorological and oceanographic patterns operating at a large scale and on the local one.

Sea Surface Temperature (SST; ºC) data was obtained from the National Oceanographic and Atmospheric Agency (NOAA) "High-resolution Blended Analysis of Daily SST and Ice" (https://psl.noaa.gov/data/gridded/data.noaa.oisst.v2.highres.html, Huang et al., 2021). These data have a daily frequency and spatial resolution of 0.25º x 0.25º and extend from 1981 to 2021 (inclusive). Time series were averaged to obtain monthly values for each pixel. Finally, they were spatially averaged to obtain SST (ºC) time series corresponding to those red rectangles named in Figure 1 as POR (Southern Portugal), GC. C (Gulf of Cádiz, Coastal area), GC. O (Gulf of Cádiz, Open Sea), Alb. C (Alboran Sea, Coastal area), and Alb. O (Alboran Sea, Open Sea) These areas were selected as representative of the environmental conditions affecting the sardine stocks sampled both in the Gulf of Cádiz and in the Alboran Sea. Because of the large mobility of this species, the regions where we collected environmental data represented both coastal and open sea environmental conditions.

Surface chlorophyll concentrations ($mg \cdot m^{-3}$) were obtained from the NASA (National Aeronautics and Space Administration) Ocean Colour web site (https://oceandata.sci.gsfc.nasa.gov). These data have a monthly frequency, and a spatial resolution of 0.083º x 0.083º. The final data set used corresponds to the MODIS and SEAWIFS sensors and the final time series extend from 1997 to 2021 (both included). Monthly time series were spatially averaged to obtain surface chlorophyll concentration time series (Chl; $mg \cdot m^{-3}$) at the same areas (red rectangles) used for SST.

To infer possible spatial differences within the regions determined by the red rectangles, monthly time series of SST and Chl were also obtained at single positions marked as red dots in Figure 1 (see also the nomenclature used to name these positions in Figure 1). These positions were selected in such a way that they could reflect any west-east gradient and also reflect the influence of river runoff and the differences between coastal and open-sea regions.

To have information about the atmospheric conditions, we downloaded reanalysis monthly time series of west-east (Ux hereafter) and south-north (Uy) components of the wind ($km \cdot h^{-1}$), and precipitation rates (P) from the National Centre for Environmental Prediction/ National Centre for Atmospheric Research (NCEP/NCAR, https://psl.noaa.gov/data/gridded/data_ncep.reanalysis.derived.surface.html/, Kalnay et al., 1996). These time series extend from 1948 to 2021 (both included) and have a spatial resolution of 2.5º x 2.5º. Time series were averaged by regions (POR, GC. C, GC. O, Alb. C, and Alb. O) (red rectangles in Figure 1).

Local meteorological conditions were obtained from the Agencia Estatal de Meteorología (AEMET, Spanish meteorological agency). Hourly time series of wind intensity and direction ($km \cdot h^{-1}$) were obtained from eight meteorological stations distributed along the Gulf of Cádiz and Alboran Sea coasts: from west to east, Ayamonte, Moguer, Cádiz, Estepona, Fuengirola, Málaga, Motril, and Almería (see blue dots in Figure 1). These time series extend from 1990 to 2020 (both included) when available (some stations started operating at a later date). Daily precipitation data (P) were obtained at the same stations from the web site (https://datosclima.es), also from AEMET. These time series extend from 1990 to 2021 (inclusive).

3.2.2.2. Data processing

SST, Chl, Ux, Uy and P are scalar time series. In the case of daily time series, they were averaged for obtaining monthly values. Then, data corresponding to the same calendar month was grouped. For instance, in the case of a time series extending from 1990 to 2021, we have 32 data corresponding to January, 32 data for February, etc. Thise data was averaged, and the standard deviation was calculated. Considering a t-student distribution, a 95 % confidence interval was estimated for each mean value. The set of twelve mean values, corresponding to the twelve months of the year, represent the climatological or average seasonal cycle of the variable considered.

In the case of wind time series from AEMET stations, hourly values of wind intensity and direction ($km \cdot h^{-1}$) were available. Besides the calculation of monthly time series of both cartesian components (Ux and Uy), wind intensity and angle of provenance referred to the north were calculated for daily wind vectors.

3.3. Results

3.3.1. Reproductive period analysis: signs of a more advanced maturation in the Southern Portugal-Gulf of Cádiz

Based on visual inspection and GSI values, a high proportion of immature individuals from the Alboran (Alb) were detected in June (78.57 %), while in Southern Portugal-Gulf of Cadiz (POR-GC) only one immature individual was reported throughout the entire sampling

(Figure 2B). While individuals in Alb were starting the gonad maturation (developing stage) during October (values of 7 % in this month), in POR-GC this process was found earlier, during June (35.24 %). As no significant differences in GSI were observed among sexes for none of the locations (Kruskal-Wallis χ^2, POR-GC: 0.166, p-value = NS; Kruskal-Wallis χ^2, Alb: 0.608, p-value = NS) (see also Table S3.1), and the sample size per gonadal stage by sex was not sufficient to establish a robust comparison, analyses were performed grouping males and females in both cases. Results indicated significant differences among gonad developmental stages in GSI by area (Welch F-test, POR-GC: $F_{7,591}$ = 122.300, p-value < 0.0001***; Welch F-test, Alb: $F_{7,424}$ = 81.930, p-value < 0.0001***), and a significant interaction between area and gonad developmental phase (Welch F-test, developmental stage*area: $F_{11,1019}$ = 135.200, p-value < 0.0001***) (Figure 2A). Significant lower GSI values were therefore quantified in developing individuals in POR-GC compared to those of the Alb, reaching similar values once fish were able to spawn. Nevertheless, a higher percentage of spawning capable fish were observed in October in the case of Alb than in POR-GC, finding also actively spawning individuals in the former (16 %). However, in November we did not detect individuals at any of these two reproductive statuses in Alb. In February, it was shown a high rate of active spawners in both areas. Nevertheless, some individuals started the regression period in POR-GC (8.79 %) in this month, which did not occur in Alb until April.

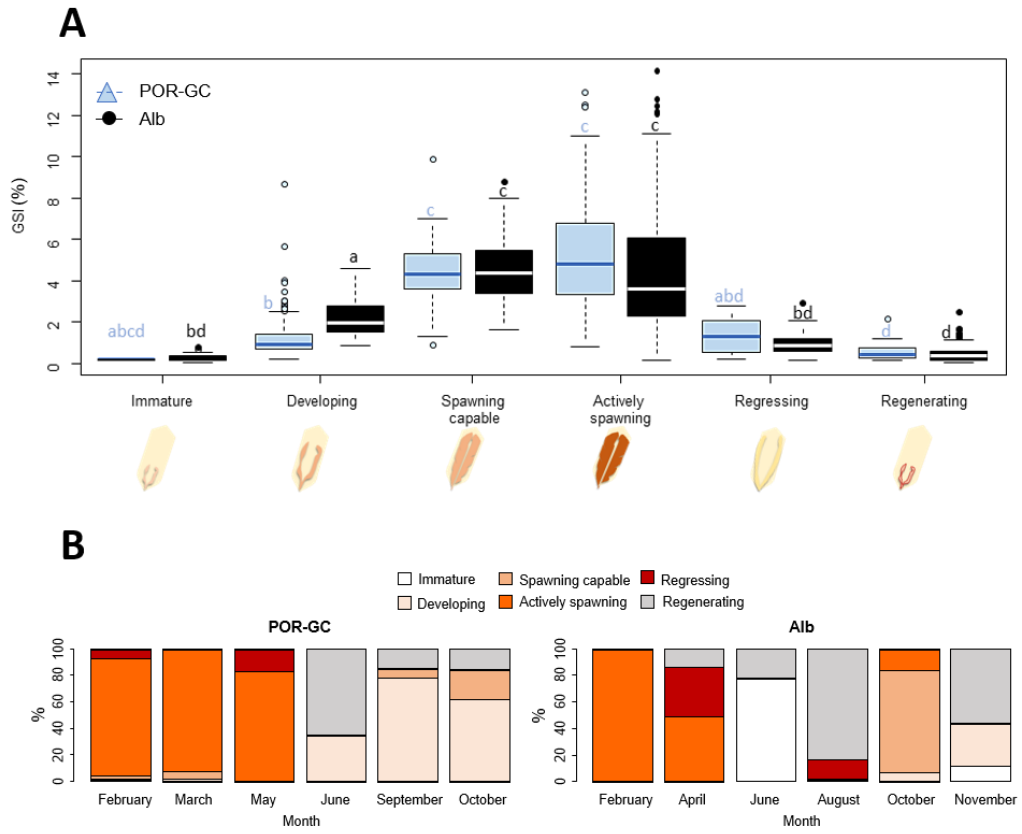

Figure 2. Reproductive analysis of European sardine (*Sardina pilchardus*) along the Southern Portugal-Gulf of Cádiz (POR-GC) (Atlantic Ocean) and the Coast of Málaga, Alboran Sea (Alb) (Mediterranean Sea). **A.** Box plot of the gonadosomatic index (GSI; %) by reproductive developmental stage according to the classification of Brown-Peterson et al. (2011) in the study areas. Different letters on the graph indicate significant differences among stages and/or locations. The minimum, the maximum, the sample median (line in the boxes), the first and third quartiles, and the outliers (○) are showed. **B.** Individuals (%) at each reproductive developmental stage by month per location.

3.3.2. Relative condition and energy storage: larger lipid reserves throughout the year in the Southern Portugal-Gulf of Cádiz area

The total body length (L_T) of the sardine specimens in POR-GC was 17.73 ± 1.30 cm (mean ± SD) (from 13.5 to 22 cm), while in the Alb it was 15.97 ± 3.14 cm (from 9.1 to 23 cm). Total body weight (W_T) was 49.26 ± 13.14 g and 38.59 ± 21.59 g for POR-GC and Alb, respectively. Thus, POR-GC sardines presented larger values for both measurements (t = -12.027, p-value < 0.0001***; t = -9.867, p-value < 0.0001***, for both length and weight, respectively).

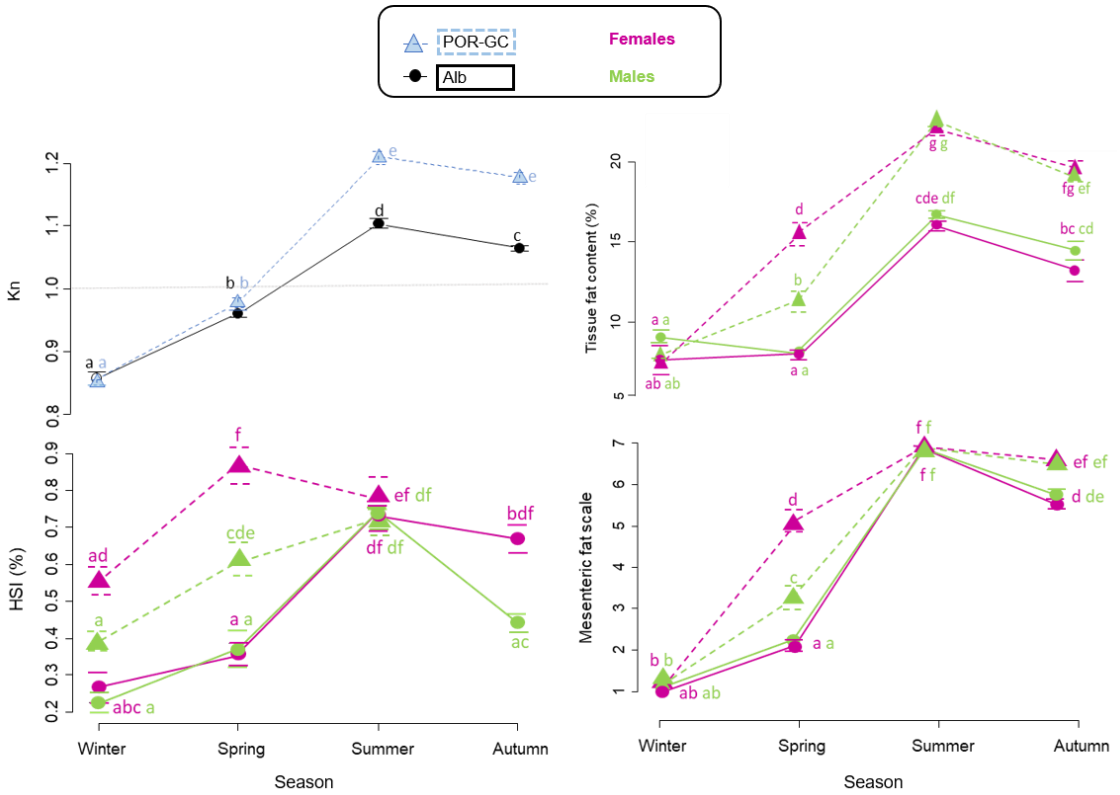

Figure 3. Energetic condition indexes of European sardine (*Sardina pilchardus*) along the Southern Portugal-Gulf of Cádiz (POR-GC) (Atlantic Ocean) and the Coast of Málaga, Alboran Sea (Alb) (Mediterranean Sea). Average ± standard error (SE) of the relative condition factor (Kn), tissue fat content (%), mesenteric fat, and hepatosomatic index (HSI; %) by season, area and sex are shown. Different letters on the graphs indicate significant differences among season, sex and/or locations. Sexes were represented separately when significant differences (p-value < 0.05*) were found within the same locality for the corresponding parameter.

For Kn, as no significant differences were observed among sexes for any of the locations (Kruskal-Wallis χ^2, POR-GC = 0.040, p-value = NS; Kruskal-Wallis χ^2, Alb = 0.318, p-value = NS), males and females were analysed together (Table S3.1). The relative condition index of the specimens was minimal during the winter and began to recover during the spring similarly in the two areas (Figure 3). However, the marked increase in Kn that occurred in summer was significantly more pronounced in POR-GC specimens than in those from Alb. These differences between areas remained significant during the beginning of the decline in condition in autumn. Moreover, average Kn in POR-GC was maintained from summer to autumn, when in Alb, the decline from summer towards autumn was significant.

General differences between sexes were detected for tissue fat content (Kruskal-Wallis χ^2, POR-GC = 4.003, p-value < 0.05*), mesenteric fat (χ^2, POR-GC = 14.919, p-value < 0.05*), and HSI (Kruskal-Wallis χ^2, POR-GC = 30.815, p-value < 0.0001***) for POR-GC individuals (analysis by season in Table S3.1), while sex differences were only observed for

HSI in Alb samples in autumn (Figure 3 and Table S3.1). Tissue and mesenteric fat content values were the lowest during winter for both locations, while in spring they were significantly higher in the specimens from the Atlantic (POR-GC). This coincided with the detection of differences between sexes for both parameters in this area, higher in females. In the case of mesenteric fat, it reached similar values among locations during summer. Regarding HSI, lower values were also observed during winter for both areas, and similarly to fat indexes, differences appeared in spring with POR-GC showing higher values. During this season, females of this area presented the highest figures. In the Alb, similarities regarding sex were established until autumn, when females mean value was significantly above the one of males.

3.3.3. Oceanographic and meteorological characterisation

3.3.3.1. SST and surface chlorophyll

Figure 4 shows the average SST (ºC) and Chl (mg·m^{-3}) concentrations for four months of the year (January, April, July and October), each representing one season (i.e., winter, spring, summer and autumn, respectively). Surface temperature was lower in the northern Alb coast than in the coast of POR-GC area for most of the seasonal cycle. The only exception occurred during the summer season, when warmer surface waters occupied both regions. Surface temperatures in the area surrounding Cape San Vicente (SV) were higher than those in the coastal waters of the GC and the northern Alb Sea during winter months, and cooler for the rest of the year. It cannot be established a correspondence between the spatial pattern of the SST seasonal cycle, and that of the chlorophyll concentrations, at least from this large-scale description. Regardless of the time of the year and the values of the water temperature, the coastal waters of the GC seemed to be the most productive ones. The highest chlorophyll concentrations in the Alb Sea were observed in coastal waters to the northeast of Gibraltar and to a lesser extent in the Málaga Bay. Notice that the shape of the Atlantic Current, surrounding the anticyclonic gyre in the western Alb Sea, can be perceived from the chlorophyll concentration distribution in April, July, and October. In the Portuguese waters, the most productive areas were found around SV during summer months. The highest chlorophyll values were observed between SV and Cape Santa María (SM) for the rest of the year.

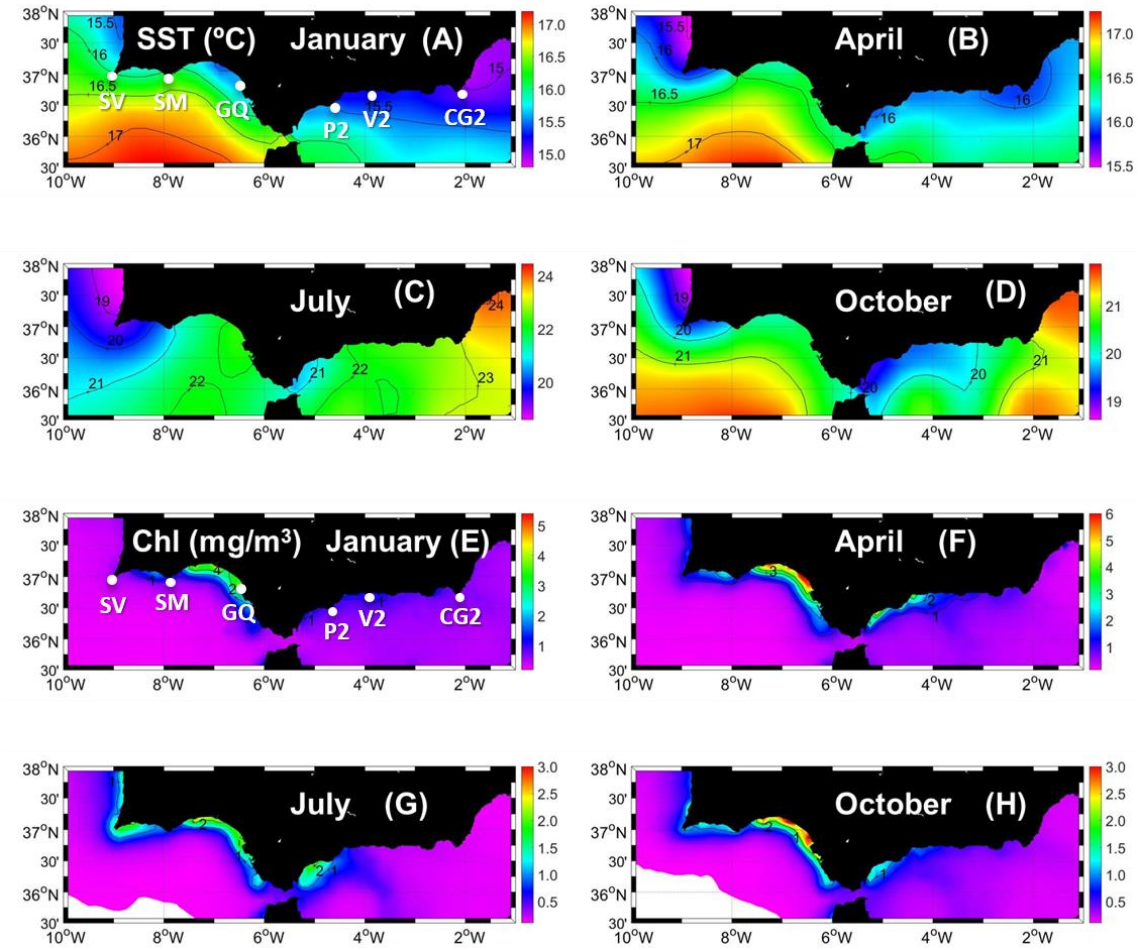

Figure 4. Maps of the mean Sea Surface Temperature (SST; ºC) (**A, B, C, D**) and mean chlorophyll concentrations (Chl; mg·m^{-3}) (**E, F, G, H**) of one month per annual season along the Southern Iberian Peninsula. SST data was daily taken from 1981 to 2021 and Chl values had a monthly frequency, obtained from 1997 to 2021. Some stations have been added to make the map easier to understand: Southern Portugal-Gulf of Cádiz stations - SV: Cape San Vicente, SM: Cape Santa María, GQ: in front of the Guadalquivir River mouth; Alboran stations - P2: Cabopino inshore, V2: Vélez inshore, CG2: Cabo de Gata inshore.

Figure 5 shows the climatological seasonal cycles of SST and Chl for the five areas presented in Figure 1 (POR, GC. C, GC. O, Alb. C, Alb. O). SST cycles showed a clear longitudinal gradient with the coldest waters in the POR sector (west), increasing towards the east. The Chl seasonal cycles also showed clearly that the most productive waters correspond to the coastal waters of the GC (GC. C - G, GQ, and M in Figure 5). This figure also evidences an important difference in the seasonal Chl cycle between the waters of the GC and Alb Sea, and those of POR. In the former cases, the maximum values were reached during the end of winter or beginning of spring, and then they started to decrease to minimum values in summer. Then, the productivity of surface waters increases again during

autumn months. In the case of POR waters, there was a secondary maximum during summer months (see Figure 5E, F).

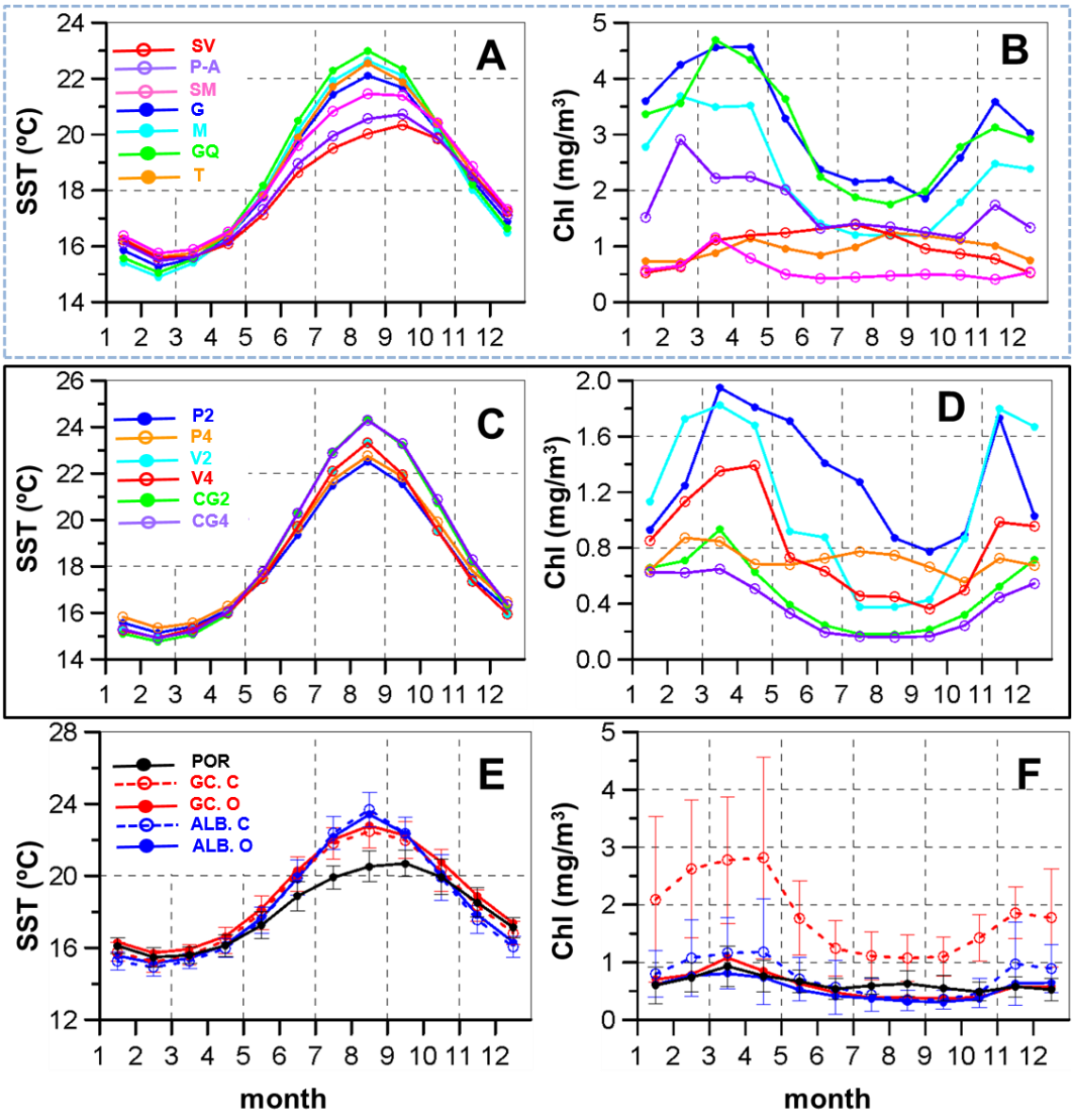

Figure 5. Mean Sea Surface Temperature (SST; ºC) and mean chlorophyll concentrations (Chl; mg·m^{-3}) by month along the study areas. **A, B.** Mean SST and Chl values for Southern Portugal-Gulf of Cádiz (POR-GC) (Atlantic Ocean). **C, D.** Mean SST and Chl values for the Coast of Málaga, Alboran Sea (Alb) (Mediterranean Sea). **E, F.** Summarised comparison of mean ± SD SST and Chl values in the study areas (see Figure 1 to identify the locations of the data extraction). SST data was daily taken from 1981 to 2021 (inclusive) and Chl values had a monthly frequency, obtained from 1997 to 2021 (both included). Southern Portugal-Gulf of Cádiz stations - SV: Cape San Vicente, P-A: Portimao-Albufeira, SM: Cape Santa María, G: Guadiana River mouth, M: Mouth of Rivers Tinto and Odiel, GQ: in front of the Guadalquivir River mouth, T: Trafalgar; Alboran stations - P2: Cabopino inshore, P4: Cabopino offshore, V2: Vélez inshore, V4: Vélez offshore, CG2: Cabo de Gata inshore, CG4: Cabo de Gata offshore).

Nevertheless, this spatial variability observed on a broad scale (red rectangles in Figure 1) can mask some small-scale features of great importance. Figure 5A, B shows the SST and Chl seasonal cycles for three positions within the POR area (see Figure 1): SV (Cape San Vicente), P-A (Portimao-Albufeira) and SM (Cape Santa María). As already mentioned, the P-A region (located between SV and SM) showed the highest chlorophyll concentrations all the year round, with maximum values during winter. The SM cycle was quite similar to that depicted for the GC and Alb Sea, while that for SV was completely different, with maximum values during the summer season. This latter area also exhibited the lowest temperatures. Monthly variations for GC and Alb areas are also presented in Figure 5. The highest chlorophyll concentrations were observed at G (close to the Guadiana River mouth), M (close to the mouth of rivers Tinto and Odiel), and GQ (in front of the Guadalquivir River mouth). The concentrations at the offshore positions (SP, GD, which together make up GC. O, Figure 5F) were much lower. Notice that the seasonal cycle at the coastal location T was different from the rest of positions and had much lower values than those observed at G, M, and GQ (Figure 5B). In the case of the Alb Sea, the highest chlorophyll concentrations were observed at P2 (Figure 5D), coinciding with the lowest temperatures. Note that the lowest SST of both areas (GC and Alb) was observed in the coastal point P2, in the north-western Alb Sea (Figure 5C). Nevertheless, these values in chlorophyll were lower than those observed in the GC (notice the different scales in the y-axes). Relatively high Chl concentrations were found at V2 and V4, whereas these values considerably decreased at the offshore point P4, and at the eastern boundary of the Alb Sea (CG2 and CG4).

3.3.3.2. Wind and precipitation rates

Figure 6 shows the wind intensity ($km \cdot h^{-1}$) (black lines), Ux component ($km \cdot h^{-1}$) (red lines) and Uy component ($km \cdot h^{-1}$) (blue lines) of the wind in the areas named as POR (6A), GC (6B) and Alb (6C). Ux wind component is positive when directed towards the east (westerly winds) and Uy component is positive when directed towards the north (southerly winds). Precipitation rates (mm) are represented by green lines.

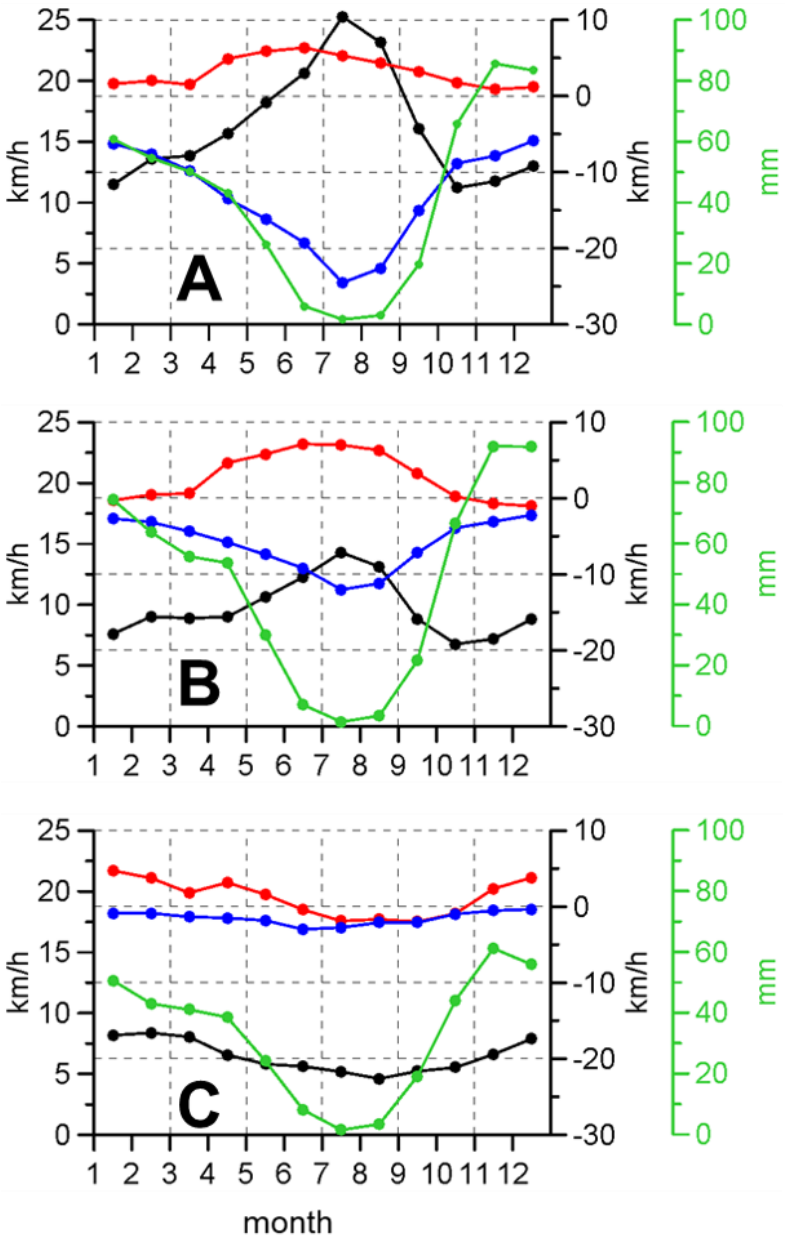

Figure 6. Monthly time series of the wind intensity, west-east (Ux) and south-north (Uy) components of the wind, and precipitation rates (P) in the Portuguese Coast (POR) (**A**), Gulf of Cádiz (GC) (**B**), and Alboran Coast (Alb) (**C**). Wind intensity in km·h^{-1} (black lines and left y-axis); west-east component in km·h^{-1} (Ux, red lines and right y-axis); south-north component in km·h^{-1} (Uy, blue lines and right y-axis); precipitation rates in mm (green lines and green y-axis).

Precipitation rates showed a longitudinal gradient with higher annually accumulated precipitation values in POR (500 mm·year^{-1}) and GC (561 mm·year^{-1}), and lower ones in the Alb Sea (390 mm·year^{-1}). Besides this difference in the annually accumulated precipitations, the seasonal cycles of the three areas had a similar form, with precipitations rates increasing from October, and maximum values at November/December. Monthly precipitation values

still remained at relatively high values until April, whereas very low values were observed from May to September.

The seasonal wind cycles also showed spatial variations, with mainly the Alb Sea differing from the other two areas. The first difference was observed in the wind intensity (black lines and left y-axis in Figure 6). Maximum intensities were observed during summer months (May to September) at POR and GC. On the contrary, the lowest intensity was observed from May to October in Alb. It is worth noting that the highest values corresponded to the POR area. The second difference concerns the south-north component of the wind (Uy, blue lines). This component was negative during the whole year, indicating the prevalence of northerly winds. Nevertheless, the amplitude of this cycle was larger at GC and mainly in the POR sector, where strong northerly winds prevailed from May to September as a result of the summer intensification of trade winds. The behaviour of the west-east component of the wind was also clearly different in both sides of Gibraltar. Whereas westerly winds prevailed throughout the year at POR and GC, with an intensification of this component from May to October, the opposite behaviour was observed in Alb. Wind blew from the west from November to May, but there was a reversal to easterly winds from June to October (red lines in Figure 6). The characterisation of the local meteorological variables from the local meteorological stations (AEMET) showed a clear trend in the cycle of the wind direction and intensity and in the precipitation rates. Although these local cycles are similar to those inferred for the larger geographical areas, obtained from reanalysis data, there are some local effects (e.g., differences appeared between Ayamonte and Cádiz stations). These local differences made it more difficult to extract conclusions about the general meteorological conditions in the Gulf of Cádiz and in the Alboran Sea. The biological data analysed in this work corresponded to years 2019 to 2021. The length of these time series does not allow us to perform any kind of multivariate analysis in order to relate the variability of the sardine condition at the different regions with the variability of the environmental variables described above. This is the reason why we have focussed on the average seasonal cycle that should highly influence the seasonal cycle of sardine growth and reproduction. Nevertheless, for the completeness of the work, Figures S3.2, S3.3 and S3.4 as supplementary material show the values of SST (ºC) and Chl concentration ($mg \cdot m^{-3}$) (Figure S3.2), wind intensity ($km \cdot h^{-1}$) and components Ux ($km \cdot h^{-1}$) and Uy ($km \cdot h^{-1}$) of the wind (Figure S3.3), and the precipitation rates (mm) (Figure S3.4), from one year before the starting of the biological sampling (2018) until the beginning of 2022. The climatological seasonal cycles are superimposed on the 2018-2021 time series for comparison.

3.4. Discussion and conclusions

3.4.1. Environmental patterns and annual energetic condition and health status

European sardine presented seasonal variability in the relative body condition index (Kn) at local level, as well as differences when comparing samples from the two studied locations at certain times of the year. We can observe that the highest values of all the studied indexes mainly corresponded to summer, when sardine is in better condition prior to its investment in reproduction during the autumn-winter months (Basilone et al., 2021; Ganias et al., 2014; Mustać & Sinovčić, 2009). This pattern of energy distribution throughout the year is common in sardines in the Mediterranean, being reported in previous studies (Albo-Puigserver et al., 2017; Pethybridge et al., 2014). The peak in condition during the summer is related to its recovery, matching with the abundance of spring food resources and prior to its investment (Figure 5B, D, E). In fact, phytoplankton are particularly important during spring and summer for sardines living in upwelling regions off the Iberian west coast (Garrido et al., 2008a).

In autumn, the decrease of mean Kn until reaching the low winter values could be explained by reserves used for gonad growth and maturation, as well as the pressure on the digestive tract of fish, which can limit food consumption (Amenzoui et al., 2006). Notwithstanding, sardines continue to feed throughout the entire spawning season to maintain the required, although low, level of energy reserves in all tissues (Zorica et al., 2019). These trends were also reflected in the energy reserves indexes (especially, in the tissue fat content, but also, in the mesenteric fat) as well as in the liver size relative to eviscerated weight or HSI, except in females from the Southern Portugal-Gulf of Cádiz (POR-GC), which showed a marked increase in the HSI from the spring months (Figure 3).

Kn mean value of individuals from POR-GC significantly exceeded Alboran's (Alb) index in summer and autumn. Furthermore, POR-GC sampled population presented in general larger values of tissue fat content, mesenteric fat scale and HSI than Alb individuals, except for winter for the three parameters, and summer for mesenteric fat and HSI, similar to the Alb. Although many uncertainties arise regarding sardine genetic population structure along the Iberian Peninsula, greater genetic similarity has been attributed between the Atlantic and Alboran stocks than between the latter and other areas of the Mediterranean (Caballero-Huertas et al., 2022a). However, phenotypic plasticity could reflect either a difference in the total energy flux through a population under different environmental conditions or a genuine alteration in the proportion of energy allocated to

each competing demand under different environmental conditions (McManus & Travis, 1998), as our results might reflect.

Thus, as differences in the measured biological variables could be influenced by the local and regional environmental conditions (Fernández-Corredor et al., 2021), we wanted to characterise the two study areas to obtain a global vision of the potential oceanographic and meteorological factors that may affect sardine condition and health status. The presence of cold waters close to coastal areas, or in the inner sector of cyclonic eddies, usually indicates the upwelling of nutrient-rich deep waters. This, in turn, enhances phytoplanktonic blooms, increasing the food availability for small pelagic species such as sardines (Somarakis et al., 2006). The results presented in this work showed these features, but they also indicated that the productivity of those waters in POR-GC and Alb are controlled by more factors than simply the wind induced or eddy induced upwelling of deep waters. In the westernmost area (POR), the seasonal cycle of chlorophyll concentration was similar to that observed in the other two areas (GC and Alb) when the whole POR region is considered, with maximum values during the end of winter. The only difference was a secondary maximum during summer (Figure 4F). Our results suggest important differences produced by distinctive mechanisms. The maximum chlorophyll concentrations were observed in winter in the P-A area, to the east of Cape San Vicente, whereas the coldest waters (Figure 5) and very likely the strongest upwelling occur at Cape San Vicente during the summer months (Figure 6). These results point to the intensification of trade winds during summer as an important factor increasing primary production from May to September in the area surrounding SV, months in which European sardine begins to feed intensively (spring-summer) to storage lipid reservoirs. This may be reflected as a higher condition and lipid reserves in stocks of the POR-GC. Nevertheless, other factors operating during winter time at P-A are more important. It should be noticed that the river Arade is close to this location, and precipitations were more intense from October to May in this area (Figure 6A), favouring large discharges. The influence of river discharges as a main factor modulating primary production in the GC is confirmed by the analysis of those locations extending from the river Guadiana (G) to Trafalgar (T, Figure 5) and by comparison with the nearby Alboran Sea, which presented much lower chlorophyll concentrations even when the wind favours upwelling processes. In this sense, the importance of upwelling has been observed in terms of enhancing primary production, although it may have a secondary role compared to other factors such as riverine input and runoff (Bonanno et al., 2016). Moreover, in the GC, from G to T, the Ux component of the wind, which should induce upwelling, had minimum values during winter and autumn (Figure 6B), but chlorophyll concentrations reached maximum values during these months. Based on this, we

hypothesized that the main fertilizing factor in POR-GC is linked to the discharges of rivers Guadiana (G) and Guadalquivir (GQ), in the Spanish sector, and Arade, in the Portuguese one. Evidence of the influence of riverine inputs on the productivity of small pelagic fish has been provided in several studies, which support our conception (Bonanno et al., 2016; Feuilloley et al., 2020; Lloret et al., 2004; Vargas-Yáñez et al., 2020; Verón et al., 2020). This hypothesis is also supported by two other facts. First, the maximum chlorophyll concentrations were observed between autumn and the beginning of spring, when precipitations reached the highest values (Figure 6), and second, such concentrations were much lower and had a different seasonal cycle in the nearby T location which is in the same area but it is not influenced by any river. Thus, direct food intake along the mentioned period (i.e., from autumn to spring) could be reflected in increased hepatic mass, which exerts a positive effect on individual ovarian mass and fecundity (Somarakis et al., 2006), and that may explain the differences in winter and, more pronounced and significant, in spring in HSI between POR-GC and Alb and, especially, in female at the Atlantic Ocean.

Coastal temperatures were colder in the Alb from autumn to spring, when the prevailing direction of the wind was from the west, favouring coastal upwelling (Figure 6C). Besides this fertilizing mechanism, the area between Gibraltar and Fuengirola (see Figure 1 for locations) exhibited high chlorophyll concentrations during the whole year, including the summer season when the winds shifted to easterlies in Alb, inhibiting the wind-induced upwelling. This is caused by two factors: the first one is the proximity of the Strait of Gibraltar where intense mixing occurs because of internal tides and the dynamics of the Atlantic Current flowing into the Mediterranean Sea (Echevarría et al., 2002; Huertas et al., 2012). The second one is the presence of a semi-permanent cyclonic eddy to the south of Estepona (see Figure 1), producing the upwelling of sub-surface waters independently of the wind direction (García-Martínez et al., 2019; Vargas-Yáñez et al., 2017, 2019). These two factors have a limited spatial scope, and do not reach the area which extends from Málaga to the east. For this reason, the primary production in this latter area decreases during the summer months, when the wind shifts to easterly, which would be reflected as a lower lipid content in the muscle in summer in the Alb sardine compared to individuals from POR-GC (Pethybridge et al., 2014), despite the fact that mesenteric fat reached similar values.

Finally, it should be considered that the lowest chlorophyll concentrations corresponded to CG2 and 4 (Figure 4. See also Figure 1 for the location of these stations), simply indicating the west-east oligotrophic gradient in the Mediterranean Sea, and also to the location P4. This latter station is close to P2, where the highest chlorophyll concentrations of the Alb Sea were found. The reason seems to be, according to the existing literature (García-Martínez et al., 2019; Vargas-Yáñez et al., 2017, 2019; Viúdez et al.,

1996,1997), that P4 is offshore and already under the influence of the oligotrophic waters in the inner part of the anticyclonic gyre that usually occupies the Western Alboran Sea.

3.4.2. Reproductive cycle along the Southern Iberian Coast: the migration hypothesis and its potential relationship with the energetic condition

Increases in sardine condition and fat reserves during the summer, when more food is available thus, higher feeding intensities, suggest that variations in reproductive traits may have been caused by environmentally driven changes in food availability (Silva et al., 2006). However, lipid storage may not be used directly to promote egg production or gonadal growth, but rather to provide energy needed for metabolism, permitting energy from food to be used for egg maturation (Somarakis et al., 2006). Thus, the analysis of curves showing the seasonal fat content could already be a first indication of potential differences in the reproductive cycle in sardines on both sides of the Strait of Gibraltar. In addition to the reserves destined for reproduction, there are other factors that seem to be associated with the spawning seasonality, as water temperature, since it has been assumed its preferences for spawning at 14-15 °C and avoidance for temperatures below 12 °C and above 16 °C (Ganias et al., 2007; Ganias, 2009; Stratoudakis et al., 2007).

Despite the fact that other works pointed out the similarities in the reproductive cycle of the two study areas (Stratoudakis et al., 2007), we reported hints of earlier maturation in sardines from POR-GC (Figure 2B), which, as already mentioned, presented a higher tissue fat content at the intensive feeding season (i.e., summer). Moreover, it nearly coincided with the lowest temperatures recorded in the south of the Iberian Peninsula in the area SV-SM in July (Figure 4C). However, individuals able to spawn were not observed until February in POR-GC, unlike in the case of individuals from Alb, which started the gonadal development earlier-along October, but also spawning capable and active spawners were found in this month, maybe linked to a drop in temperatures along this coast (Figure 4D). Given these results, and although we lack data for some months (among other reasons, due to fishing closures), potential explanations could be provided. The first is that Atlantic (POR-GC) individuals entered in Mediterranean waters when they were able to spawn, reason why high percentages of spawning capable and active spawners were present in October in Alb whereas no spawning capable nor developing individuals were found during August, the previous month when data was available. It seems to be difficult that both processes of developing and spawning capability occurred in the month of September (lacking data)

until reaching the values observed in October for the local individuals in Alb. Thus, one of the reasons of this migration of spawning capable and active spawner individuals from POR-GC may be this drop in local temperature in October in the Alb area. Also, stable currents as the inflow of Atlantic water in the Mediterranean Sea through the Strait of Gibraltar foster the exchange of species among adjacent basins (García-Lafuente et al., 2021), which would be promoting sardine mobility towards Alb in some way.

However, and additionally, studies pointed out the existence of a featured spawning area located in Alb. Important sardine nursery grounds along the northern coast of the Alb Sea, Málaga and Almería Bay (Quintanilla et al., 2020; Rubín et al., 1997; Würtz, 2010) provide shelter from the large-scale westerly wind flow (Würtz, 2010) that occurs in the area during the beginning of the reproductive season. Besides, the presence of the Alb gyre could be causing larvae retention (Naciri et al., 1999) and hindering dispersal and migration (Bacha et al., 2014) of the juveniles, potential reason to explain why we found a high percentage of immature individuals during June in this area while they were absent in POR-GC in the spring months. Bernal et al. (2007) characterised zones of significant spawning preference in the POR-GC region from 1985 to 1995. It has been shown that winters with strong southward winds and weak but consistent upwelling events along the Portuguese coast led to poor recruitment of sardines in the following months due to a large offshore transport of eggs and larvae when their spawning season takes place (Horta e Costa et al., 2014; Santos et al., 2001). However, our results reflected a prevalence of westerlies along the year in this area. Thereby, the coupling between spawning and circulation is particularly beneficial under westerly winds, when productivity in the eastern shelf is likely enhanced and the plankton is confined within the cyclonic cell (Lafuente & Ruiz, 2007). Nevertheless, it has to be taken into account that changes in the circulation patterns could affect the physical mechanisms of concentration and retention for a particular area (Mercado et al., 2007), which has been described and modelled in POR-GC, with a bimodal pattern of anticyclonic circulation in spring-summer that changes at a certain time in winter (sardine reproductive period) to cyclonic (Lafuente & Ruiz, 2007). Thus, it could be probable that potential migrations from POR-GC towards Alb were caused due to the greater success in spawning and recruitment in this area, which would be directly related to the behaviour of capital breeders, which spawn and feed in separate areas during different seasons (McBride et al., 2015).

The existence of a common spawning ground in fish for different subpopulations has been supported by various hypotheses, among which we can find the patchy population hypothesis, with the absence of a 'source population', or the metapopulation hypothesis, which considers that subpopulations can have autonomous (and eventually divergent)

lives. Each subpopulation is mostly linked to a particular spawning area, but a small, albeit significant, quantity of adults may move from one spawning area to another (Gerlotto et al., 2012; Kritzer & Sale, 2004; Ovaskainen & Hanski, 2004). Contrary to the patchy population, the existence of a source population is one key characteristic of the metapopulation (Gerlotto et al., 2012). In the context of a metapopulation, it has also been suggested a migrant hypothesis for the clupeid Atlantic herring, in which the progeny of a given local population do not necessarily recruit to their natal population, but may become migrants, contributing to an adopted population having the same or a different reproductive season. Population affinity is established at the time or first maturation and is fixed for all subsequent spawning by adhering to an annual maturation cycle (McQuinn, 1997). Moreover, similar scenarios to the one proposed have been reported for the fully recruited E. sardine population in the central Mediterranean Sea during autumn and winter, with migratory behaviour for spawning purposes (Basilone et al., 2021). Also, and as an example, in the Adriatic Sea (Mužinić, 1973; Škrivanić & Zavodnik, 1973) authors suggested that spawning grounds are located in the areas with optimal biological conditions, where the hydrographical equilibrium is established between two types of sea water, the Mediterranean and the Alpic (Škrivanić & Zavodnik, 1973). Sardine migration to these locations is triggered by temperature and then, the return of fish schools to feeding areas takes place, resulting in limited mixing of sardine stocks inhabiting various regions of the Adriatic Sea (Škrivanić & Zavodnik, 1973). This would explain why a certain percentage of fish that have already spawned (regressing stage) was recorded in POR-GC in February, and then, registered again in May, due to differential spawning between individuals and the later return to their area of origin. Thereby, under any of these models, the genetic similarities (Antoniou et al., in press; Caballero-Huertas et al., 2022a) between sardines in the study locations could be explained. The oceanographic processes occurring off the Strait of Gibraltar have been demonstrated to act as barriers to gene flow for many fishes, although studies suggest that for some species (among them, another clupeid as the European anchovy) there are not genetically isolated stocks on both sides of it (Bacha et al., 2014; Jemaa et al., 2015), which could also be occurring in sardine due to the reproductive patterns of migration suggested. However, and returning to the example of the anchovy, other investigations have proposed the Alboran stock as a population independent of the Gulf of Cádiz and the rest of the Mediterranean, being located between the barriers of the Strait of Gibraltar and the Almeria-Oran oceanographic front (Viñas et al., 2014). This lack of consensus is a common circumstance among pelagic fish taxa, since the study of their population structure is not only limited by the resolution of genetic markers but also by their wide dispersal capabilities in all the stages of the life cycle (Caballero-Huertas et al., 2022a). Within the sardine scenario, the Almeria-Oran front has been proposed as a barrier

to its dispersal that could be held responsible for founder effects, followed by genetic drift with selection acting on the local scale driving adaptations mostly related to minimum SST (Antoniou et al., in press). Nonetheless, the Strait of Gibraltar would not be an effective genetic barrier for this species, as proposed by our hypothesis and the previously mentioned genetic structure studies on the similarity between Atlantic and Alboran individuals.

POR-GC sardines reached their maximum condition in the months around the end of summer-the beginning of autumn (Figure 3), reflected by Kn, tissue and mesenteric fat content, overlapping with an early gonadal maturation. The most advanced individuals in terms of gonad maturation stage may be those that migrate to Alb to spawn, so then in Alb we found worse condition (Kn) because those individuals that came from the Atlantic (and we may have identified as Alb individuals) spawned and, thus, a decrease of average Kn, tissue fat content and mesenteric fat is observed in this period in Alb. Meanwhile, those that remained in the Atlantic in autumn may have not yet started to spawn and, therefore, had better indicators, and similar to the ones registered in summer. In spring, it could be possible that Atlantic individuals kept coming back to POR-GC from the spawning areas in Alb, so it would be logical that Kn curve for POR-GC samples was close to Kn values of Alb individuals because general fish condition (as suggested by Albo-Puigserver et al. (2020), mostly focused on changes in protein content) had not recovered yet. Nevertheless, greater fat reserves were appreciated at this season due to the availability of feeding resources on this side of the Strait because of the environmental aspects previously mentioned. In this regard, it would be advisable to increase the sample size in future studies to be more accurate in these statements. In addition, we propose to carry out a monthly and balanced comparison in order to verify many of the hypotheses launched in this work.

Population connectivity plays a fundamental role in local and metapopulation dynamics, community structure, and the resiliency of populations to human exploitation, so detecting the connectivity in a managed area is essential (García-Lafuente et al., 2021; Muñoz et al., 2015). However, in order to confirm or discard the spawning migration hypothesis and set it within the framework of a patchy population or a metapopulation, a monthly/seasonal genetic sampling would be necessary to compare sardines from both sides of the Strait of Gibraltar over several years to check, also, if this potential migration is due to a punctual behaviour of few individuals or to a pattern that is repeated by a large percentage of the stock. In addition, this study shows that it is necessary to genetically identify the stocks in order to be able to draw the appropriate conclusions, given that there may be some confusion as to the origin of the differences (i.e., purely environmental or mix of individuals from different subpopulations) in certain parameters of condition and

reproduction. These issues require special attention in terms of stock assessment and management.

Chapter 4

From west to east: heterogeneity in the life history traits of a small pelagic fish (*Sardina pilchardus*) throughout the Mediterranean

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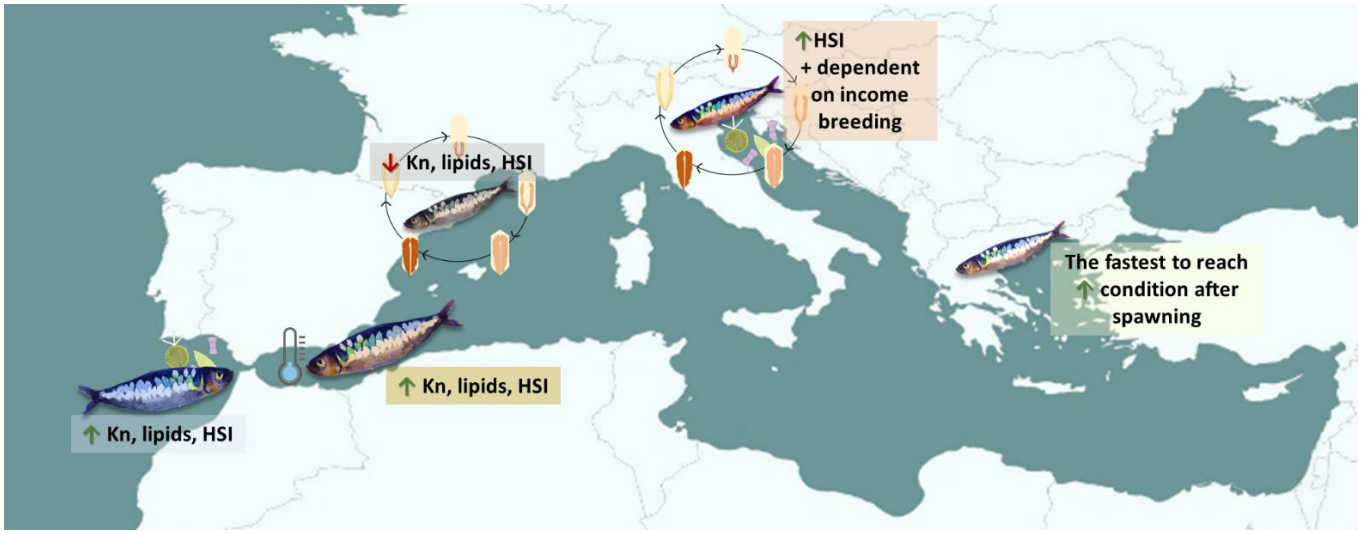

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Abstract

Small pelagic fish are key elements of the marine ecosystem and of great importance with respect to the total landing of marine species. The last decades, a decrease in biomass and catches of European sardine has been described, especially in the Mediterranean Sea, indication of a drop in the stocks' condition. Multiple causes, among them direct and indirect anthropogenic factors, seem to be converging and affecting to this ecologically and economically important species. To address this issue, it is important to identify the current variability in sardines' health status and understand the strategies for energy allocation, intimately related to the reproductive potential and recruitment success. We analysed the somatic condition through tissue and mesenteric lipid measurements, relative condition (Kn) and hepatosomatic (HSI) indices, and reproductive condition with the gonadosomatic index (GSI) in four subareas of the Mediterranean (Northern Alboran, Northern Spain, Northern Adriatic, and Aegean Sea) and of an outgroup from the Atlantic. The analyses were performed within the gonadal development framework since the translocation to reproduction may mask the state of actual energy reserves for comparison. Results revealed marked differences in health status among subareas, standing out the low condition of the Northern Spain sardines throughout the annual cycle. The uniform condition in the Northern Adriatic suggests that resources availability modulates the reproductive strategy since in this locality sardine would have a high dependence on immediate reserves, not behaving as a strict capital breeder. Moreover, similarities between Alboran and Atlantic stocks are discussed, highlighting the marked energy replenishment of the latter after reproduction compared to the Mediterranean stocks. The Aegean stock was the fastest to reach its maximum after spawning. Finally, the role of liver in sardine's energy storage is analysed considering stocks' heterogeneity. Given the divergences among stocks, we propose concrete measures for the management of the resource throughout this important water body, which could be applied to other species in similar contexts.

Keywords: Capital breeder • European sardine • Fisheries • Gonadal stage • Health status • HSI • Small pelagic

4.1. Introduction

Growing human pressures, including climate change, are having profound and diverse consequences for marine ecosystems (Doney et al., 2012). Among the impacts throughout the food web, the effects of a changing environment include variations in the life history traits of fish, which are closely linked to population dynamics (De Roos et al., 2003; King & McFarlane, 2003; Liang et al., 2014). This includes changes in the growth rate and condition, and length and age at maturation and fecundity (Devine et al., 2012; Lloret et al., 2013), which in turn are closely related to each other because of the the trade-offs between the energy invested in growth, maintenance and reproduction (Albo-Puigserver et al., 2021). In this regard, important collapses have been documented in pelagic fish in the last decades (Fréon et al., 2005) due to population alterations as a likely result of induced changes in life traits.

The Mediterranean is considered one of the most impacted seas in the world, since climate change interacts synergistically with many other disturbances (Halpern et al., 2008; Lejeusne et al., 2010), as fishing pressure. In fact, together with the Black Sea, it presented the highest percentage (62.5 %) of stocks fished at unsustainable levels worldwide in 2017 (FAO, 2020), and the second highest in 2019 (63.4 %) behind the Southeast Pacific (FAO, 2022). Moreover, it ranks among the fastest warming ocean regions (Marbà et al., 2015), favouring the periodicity of extreme events (Di Biagio et al., 2020) in combination with other drivers. In this context, small pelagic fish stocks in the Mediterranean have been suffering from population declines (Ramírez et al., 2021), and some of them have collapsed (Colloca et al., 2017). That has been mostly attributed to human activities, included the intensive exploitation throughout the decades, and also to the sensitivity of these species to environmental fluctuations (Martín et al., 2012; Van Beveren et al., 2014; Coll et al., 2019), especially related to the thermal factor that has been altered by the anthropogenic effect at global scale. The increasing temperature does not only have a direct action on the physiology and behaviour of individuals, but also in plankton productivity and available resources (Brosset et al., 2017), making it a dominant environmental driver for predicting pelagic fishery health (Lloret et al., 2021). In addition to the potential economic consequences of this event, the decrease of small pelagics in ecosystems may lead to affect all biological levels, since they act on the higher and lower trophic levels, exerting a wasp-waist control of these organisms in the system (Cury et al., 2000; Corrales et al., 2015).

The European sardine *Sardina pilchardus* (Walbaum, 1792) is a neritic small pelagic fish species with cold-temperate water affinity widely distributed in the north-eastern Atlantic areas from the North Sea to Senegal, including the Mediterranean and its adjacent

seas (Parrish et al., 1989), in which accounts for around 15-20 % of the total marine captured production (Tsikliras & Koutrakis, 2013). Its reproduction is characterised by an early maturation, many eggs per body mass, and batch spawning, common strategies to compensate for the short lifetime fecundity of the small pelagics (Ganias et al., 2014). Furthermore, its reproductive cycle coincided with that of a capital breeder (McBride et al., 2015), since it stores energy mainly during a concrete period, in this case, during the peak of production in spring-summer, and then allocates it to reproduction in autumn-winter, although the reproductive phenology of sardine stocks depends on the area (Caballero-Huertas et al., 2022b). Thereby, its reproductive strategy determines its condition throughout the annual reproductive cycle, which is also affected by environmental factors.

Studies have reported a decrease in biomass and catches in several subregions of the Mediterranean basin, associated to a slower growth, a decrease in body length, the disappearance of older individuals, and a worse body condition and health status of sardines (e.g., Sinovčić et al., 2008; Van Beveren et al., 2014; Brosset et al., 2017; Quattrocchi & Maynou, 2017; Şenbahar et al., 2020). In order to address these facts, understanding the variability in populations condition and health constitutes a principal question in fisheries research and is of special importance to management. Condition and energy storage have important implications for life history traits, such as growth, mortality, or reproduction, affecting recruitment and having an effect on population dynamics, what, ultimately, may alter pelagic ecosystem structures (Albo-Puigserver et al., 2017, 2020; Caballero-Huertas et al., 2022b).

Even though the Mediterranean is, globally considered, an oligotrophic sea, it presents considerable heterogeneity (Estrada, 1996), so that sardine stocks are affected by a differential set of climate and ocean conditions, mainly during larval development and recruitment (Leitão et al., 2014). In addition to the different productivities of the various ecosystems, the trophic status and the genetic distance among populations may be key factors in the potential diverse condition reflected throughout sardine's Mediterranean distribution (Dimarchopoulou & Tsikliras, 2022), which may also influence reproductive phenology and potential.

In this sense, there are scarce works that simultaneously recorded the variability in sardine's condition and reproduction along the Mediterranean Sea, since the studies that compared individuals from several locations involved few Mediterranean subregions (e.g., Ganias et al., 2007, Albo-Puigserver et al., 2021, Caballero-Huertas et al., 2022c), and/or analysed other biological traits as sardine's growth (e.g., Dimarchopoulou & Tsikliras, 2022) or length at maturity (e.g., Silva et al., 2006). An investigation by Brosset et al. (2017) on the

spatiotemporal variability in body status of sardine (also anchovy) in different subregions of the Mediterranean registered an asynchronous drop in condition at various locations for both species. This study was carried out with data taken along the period between 1975 and 2015, therefore it makes sense to propose new research that explores the current state of condition and health status of the sardine within its reproductive context that continue the time series. In this way, the scientific interest of this work lies in describing the present characteristics and peculiarities of each sardine stock associated with a specific area and speculating on the source and origin of the potential differences, taking into account the annual cycle of each stock due to its implication in energy status. To analyse the energy dynamics considering the concrete gonadal stage of the individuals allow to carry out a more accurate approximation to the real state of condition and its sources of change than evaluate the individuals considering the sampling months, as has already been purposed for other species (e.g., Muñoz & Casadevall, 2002; Serrat & Muñoz, 2022). Furthermore, as fishing quotas/sustainable yields should be adapted to a regional scale because of the regional variability that conditions the status of the stocks (Leitão et al., 2014), updated knowledge of these aspects throughout the Mediterranean distribution of sardine is essential for the efficient management of this resource.

The main objective of this study has been to characterise and compare the body condition and reproductive features of diverse Mediterranean stocks and the closest Atlantic population within the gonadal development framework to globally document the current health status of this species throughout a remarkable part of its distribution, and in which a greater vulnerability has been documented. Hence, our research aims to answer the following questions: is there a quantifiable variability in the current condition and energy storage of the Mediterranean sardine? If so, is this due to the different life strategies modulated by the environment? How should the management of the fishing resource act and how should the fishing policies be updated?

4.2. Materials and methods

4.2.1. Sampling collection and description of the areas

Specimens of European sardine, *Sardina pilchardus*, (N = 3101) were collected seasonally (Table S4.1) (seasons defined as winter: January, February, March; spring: April, May, June; summer: July, August, September; autumn: October, November, December) from

2019 to 2021 throughout four Mediterranean sub-areas (GSA), following the General Fisheries Commission for the Mediterranean (GFCM) delimitations established for stock assessments by commercial fisheries: GSA 1-Northern Alboran Sea, and GSA 6-Northern Spain (Western Mediterranean Sea); GSA 17-Northern Adriatic (Central Mediterranean Sea); and GSA 22-Aegean (Eastern Mediterranean Sea). An external point to the Mediterranean Sea was selected, located in the Northeast Atlantic Ocean according to the subareas and divisions of FAO fishing areas (Portuguese Waters - East (FAO fishing area division 27.9.a)) (Figure 1). Immediately after the purchase, samples were frozen at - 20 ºC, which has been demonstrated that it does not affect condition variables (Brosset et al., 2015a). Excepting 32 individuals of the total (1.03 %), all specimens in this study respected the minimum landing size for sardine (total length (L_T) of ≥11 cm)) in the Mediterranean Sea (Popescu, 2018).

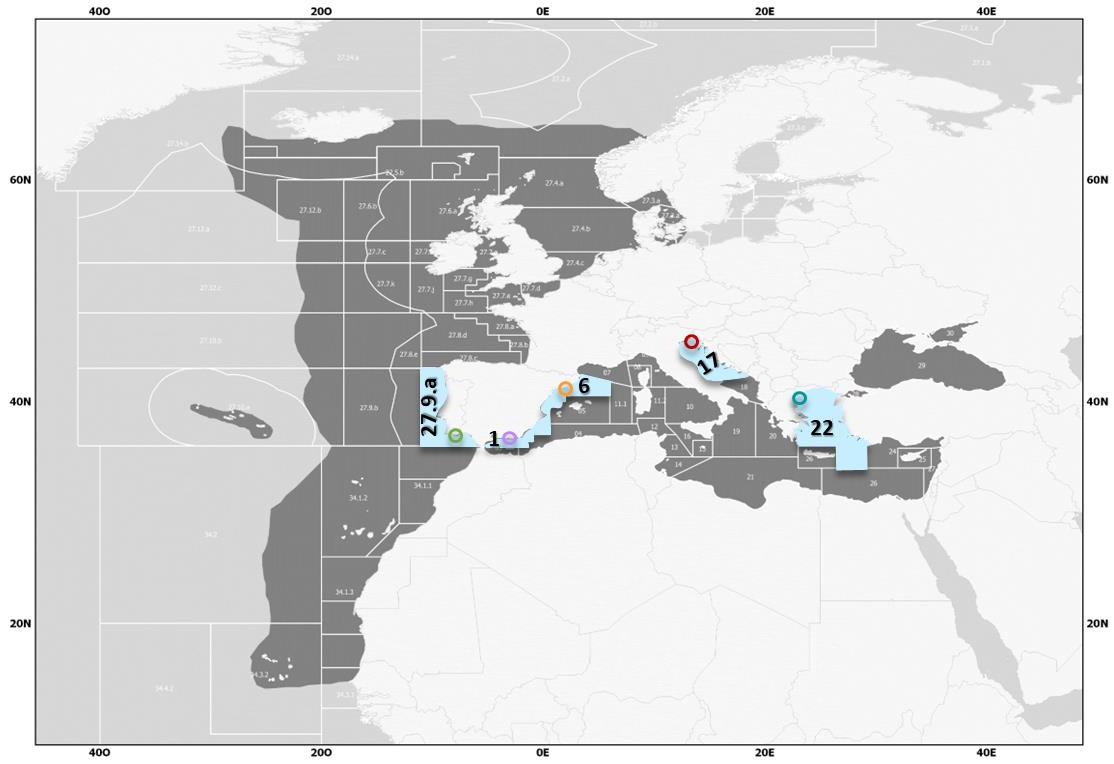


Figure 1. Geographical Subareas (GSAs) of the Mediterranean selected for the study (light blue) and approximate sample collection areas. Areas defined by the General Fisheries Commission for the Mediterranean (GFCM) as GSA 1 (violet): Northern Alboran Sea (Coast of Málaga), GSA 6 (orange): Northern Spain (Catalan Coast), GSA 17 (red): Northern Adriatic (Gulf of Trieste), and GSA 22 (turquoise): Aegean Sea (Thermaikos Gulf), and the external sampling point in the Atlantic, as FAO division 27.9.a: Portuguese Waters – East (Southern Portugal and Gulf of Cádiz).

The Northern Alboran Sea subarea (GSA 1) is in the southeast of the Iberian Peninsula, in the western Mediterranean Sea. It constitutes a transitional zone between Mediterranean and Atlantic waters, which are connected by the Strait of Gibraltar. This area does not present as much annual temperature variability as other Mediterranean areas (Figure S4.2 A). There is an intense influence of the circulation, with a strong frontal area that divides a productive sector of intense upwelling in the coastal zone, and a more oligotrophic area far offshore (Vargas-Yáñez et al., 2017, 2019; García-Martínez et al., 2019; Caballero-Huertas et al., 2022c). The Northern Spain subarea (GSA 6) is located in the North-western (NW) Mediterranean and it comprises the Catalan Sea, in which samples from this area were collected, and the Gulf of Valencia. Sea surface temperature (SST) varies significantly during the year: summers in which the water exceeds 26 ºC and temperate-cool winters (Figure S4.2 A). Except for the areas near the mouth of the Ebro River, production remains at low and constant levels throughout the year (Figure S4.2 B). The commercial exploitation of small pelagics in the area has been significant since the early 1940s, with catches dominated by sardine, probably due to its coastal distribution, and constituting one of the most productive Mediterranean sardine stocks (Palomera et al., 2007; Silva et al., 2008). The Northern Adriatic Sea (GSA 17) is located in the northernmost section of the most septentrional Mediterranean sub-basin. It is one of the major chlorophyll hot spots in the Mediterranean Sea (Figure S4.2 B), as it is influenced by the nutrient discharge from the Po River and a dozen small rivers that flow into the Adriatic Sea north of the Po River delta and in the Gulf of Trieste, with around 40 % of the chlorophyll production of the whole Adriatic (Rizzi et al., 2016). Small pelagic fishing by trawlers and purse seiners (mainly focused on anchovy and sardine) is mostly concentrated in the northern part of the Adriatic Sea (Carpi et al., 2017). The North Aegean Sea (GSA 22) shows variations in SST during the seasons, with hot summer months (Figure S4.2 A). Although its production levels are low throughout the year, except for variable increases during some summer months (Figure S4.2 B), it is more productive than the highly oligotrophic southern part, as it is influenced by Black Sea waters and large rivers, constituting one of the most important small pelagic fishing grounds in the eastern Mediterranean (Somarakis & Nikolioudakis, 2007). European sardines from the external sampling point (27.9.a) were mainly collected along the Southern Portugal and Gulf of Cádiz. In this area, sea surface temperature (SST) variations along the year are lower than in the Mediterranean Coast (Figure S4.2 A). It is characterised by a notable and continuous productivity (Figure S4.2 B) mainly due to rivers discharges (Caballero-Huertas et al., 2022c), since the effects of upwelling are reduced and occasionally occur under the influence of local westerly winds, or when upwelled waters from the west Portuguese Coast intrude over the southern shelf break (Garrido et al., 2008a). *Sardina*

pilchardus is the main target species of the purse seine fleet, contributing around 98 % of the landings in this division (Leitão et al., 2014).

4.2.2. Analysis of reproductive investment and phenology

The gonadosomatic index ($GSI = 100 \cdot \frac{W_G}{W_E}$, where $W_G \pm 0.0001$ g is the gonad weight, and $W_E \pm 0.01$ g is the eviscerated body weight) was calculated as an indirect method to estimate the reproductive effort or energy allocated to reproduction (Somarakis et al., 2004; Brosset et al., 2016). Sex and reproductive developmental stage (Figure 2) were visual and macroscopically determined, and confirmed under stereomicroscope (Zeiss binocular magnifier), to classify the gonads according to the criteria developed by Brown-Peterson et al. (2011) into the following categories: immature (sexual maturity still not reached); developing (gametes begin to develop in gonads that start to grow); spawning capable (almost ready for the reproduction); actively spawning (expelling the gametes), regressing (gonads almost empty of gametes), and regenerating (mature but reproductively inactive).

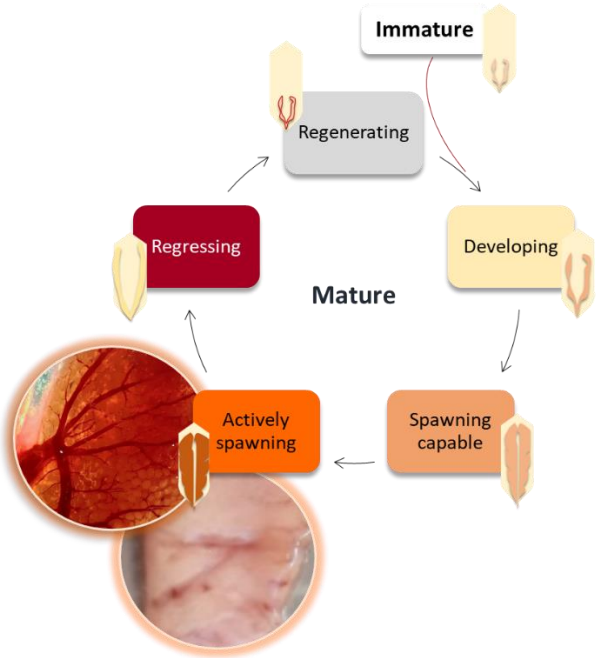


Figure 2. Reproductive cycle of the European sardine (*Sardina pilchardus*) according to the gonadal developmental stages defined by Brown-Peterson et al. (2011).

4.2.3. Somatic condition evaluation

Sardine's morphogravimetric values to estimate body condition were taken by measuring length (total length, L_T ± 0.1 cm) and weight (total body weight, W_T ± 0.01 g; eviscerated body weight, W_E ± 0.01 g). Liver (W_L ± 0.0001 g) weight was also taken to calculate the hepatosomatic index ($\text{HSI} = 100 \cdot \frac{W_L}{W_E}$). A morphophysiological approach for evaluating the general somatic condition was obtained by the calculation of the relative condition index (Kn) (Le Cren, 1951), considered as higher-than-average when Kn exceeds 1 for a given individual, and lower when it is under this value:

$$\text{Kn} = \frac{W_E}{W_r} = \frac{W_E}{\alpha {L_T}^{\beta}}$$

where W_E is the eviscerated body weight of an specimen, and W_r is defined as the predicted eviscerated weight of an individual of a given total length. L_T is the total length, and α and β are coefficients obtained by the regression line of the logarithms of length and mass (α = 0.0037, β = 3.2418). Moreover, total tissue fat content (i.e., muscle total lipids) was estimated by the average of both sides along the fish lateral line using a fish fat meter (Distell Model FM 992) (Kent, 1990) calibrated for European sardine. Besides, a visual scale for fat mesenteric reserves (Van Der Lingen & Hutchings, 2005) was used.

4.2.4. Statistical analysis

Analyses were carried out making use of R software version 4.2.1 (R Development Core Team, 2018). Differences among categories were considered as statistically significant if p-value < 0.05. Significance values were indicated as follows in the Results section: p-value < 0.05*; < 0.001**; < 0.0001***. When continuous dependent variables were involved, Shapiro-Wilk test was applied to test the assumption of normality and Levene's test was executed to prove the homogeneity of variances (Zar, 1996) in all parameters. If both assumptions were met, one-way analysis of variance (ANOVA) were performed. Conversely, when only homoscedasticity assumption was violated, data was analysed with Welch's t-test. For those parameters in which normal distribution was lacking but homoscedasticity was present, Kruskal-Wallis analysis of variance was applied. In the analyses of the indices that did not accomplish with normality and homogenous variances and data transformation was not succeed in correcting the lack of normality or heteroscedasticity, generalized linear model (GLM) was carried out. When required, multiple comparison or post-hoc tests

(Tukey's range test, Dunn's method with Bonferroni adjustment, or Games-Howell test, when corresponded) were applied to identified different categories. For qualitative dependent variables, Pearson's Chi-squared test was performed.

4.3. Results

4.3.1. Reproductive period analysis: spatial variability in reproductive phenology along the Mediterranean

Overall sex ratio by subarea (m/f) did not deviate significantly from the hypothetical distribution 1/1 in the Atlantic sampling point (χ^2 = 1.217, *df* = 1, p-value > 0.05), in GSA 1 (χ^2 = 1.715, *df* = 1, p-value > 0.05), in GSA 6 (χ^2 = 0.025, *df* = 1, p-value > 0.05), but differed in GSA 17 with a highest number of females (292/411: χ^2 = 20.144, *df* = 1, p-value < 0.000***), and GSA 22 with more males than females (335/281: χ^2 = 4.734, *df* = 1, p-value < 0.05*). Spawning capable sardines appeared during autumn in all the locations, with actively spawners in the Mediterranean subareas but not recorded in the Atlantic in this season (samples from the early autumn) (Figure 3). The highest percentage of active spawners was observed during winter in all the subareas, with more than 80 % of the individuals at this stage, although 87.56 % of the spawners in Northern Adriatic (GSA 17) were analysed in autumn. The spawning season was lengthened in time in the Atlantic and in the Atlantic-Mediterranean transition zone GSA 1, with a significant number of active individuals in both zones in spring (40.20 and 23.56 %, respectively). Gonad maturation started to be observed in summer in all the locations except in the Alboran (GSA 1), with most individuals at the regenerating stage in this locality. Few immature individuals were recorded in winter in the Atlantic, in summer in GSA 1 and GSA 17, and a larger number of them were obtained in spring and autumn in GSA 1 (5.28 and 5.94 %, respectively), in spring and summer in Northern Spain (GSA 6) (17.27 and 9.52 %, respectively), and autumn in the Aegean Sea (GSA 22) (9.55 %).

A notable drop in GSI values were seen after winter, coinciding with the decrease in the number of active spawners, until reaching the minimum values in spring and summer, recovering afterwards during autumn. When paying attention to the comparison of GSI among stocks, we could observe marked differences throughout the cycle, except for immature individuals (NS) (observable in S4.3 and in the scale of the right y-axes of Figure

3). In this regard, the lowest values in the actively reproductive stage in Northern Spanish stock should be noted. Despite these significant differences among subareas along the reproductive cycle, differences between sexes in GSI by location were especially remarkable along spring and summer, with significant female values above male's (Figure 3). Only significant higher values for male individuals were observed in autumn in GSA 22 (Kruskal-Wallis $\chi^2 = 19.906$, $df = 1$, p-value < 0.0001***).

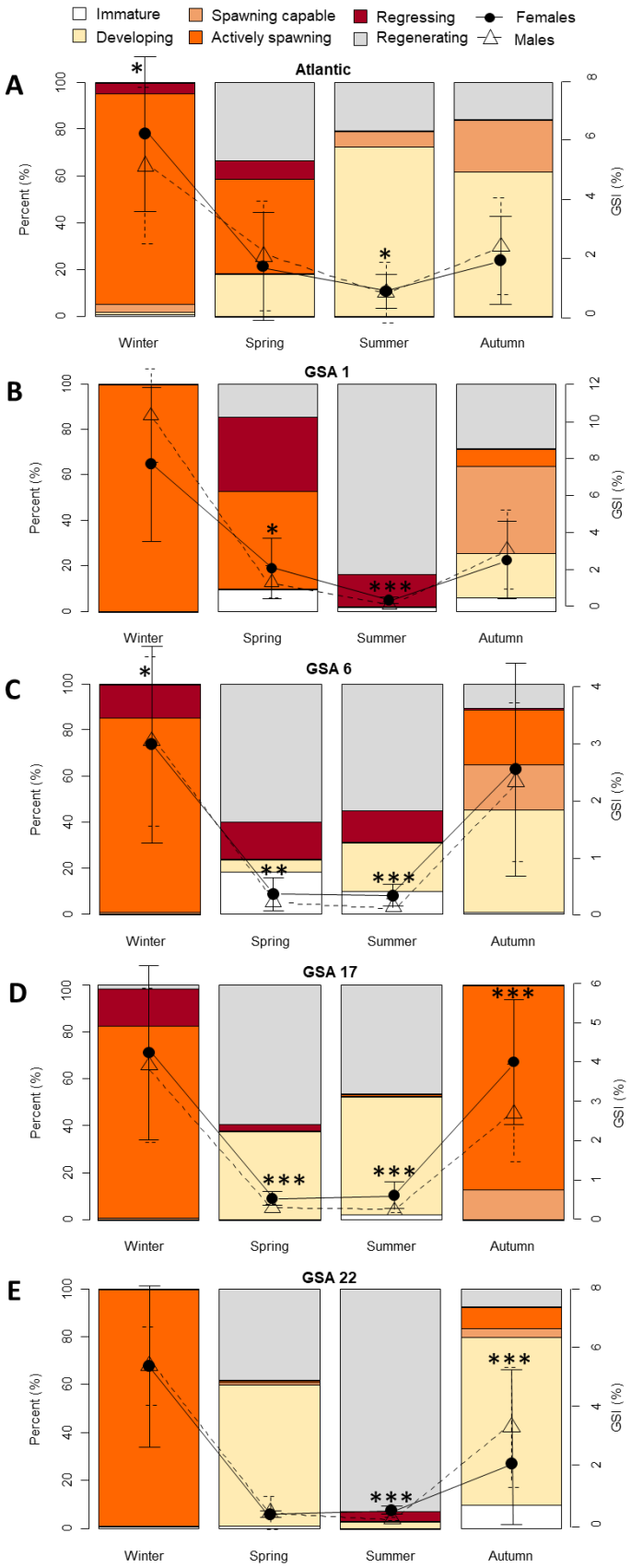


Figure 3. Seasonal reproductive analysis of European sardine's (*Sardina pilchardus*) in the Atlantic stock (**A**) (FAO division 27.9.a: Portuguese Waters – East) and four Mediterranean GSAs ((**B**) 1: Northern Alboran Sea; (**C**) 6: Northern Spain; (**D**) 17: Northern Adriatic Sea; (**E**) 22: Aegean Sea). Stages of the gonads are represented by the

percentage (%) of individuals at each reproductive developmental phase (left y-axis), and mean GSI (%) ± SD (right y-axis). Females continue line and males, dashed line.

4.3.2. Energy storage and relative condition: differences along the Mediterranean distribution according to the reproductive cycle

Taking into consideration the adult and reproductive sardines, the lowest values of tissue fat content (Figure 4A, D, G, J, M, P) were generally found during the active spawning (Figure 3 J), accompanied by the lowest values of mesenteric fat (Table S4.3). In general terms, it was recorded a continuous decrease in Kn from the developing stage towards the actively spawning phase (Figure 4E, H, K). When individuals reached the regressing phase (Figure 4N), values started to increase reaching the regenerating phase, as could be seen in the Kn medians by subregion, which exceeded the threshold value equal to 1. However, variations were detected between subregions throughout the reproductive cycle in relation to the moment in which individuals reach maximum condition and their subsequent decline. The Atlantic sardines presented high values of condition (especially reflected by the tissue fat content and Kn) in the developing and the spawning capable stages. However, during the active spawning and the regressing phases, these values were low (Figure S4.3 and Figure 4J, K, L, M, N, O) and, concretely, the lowest in Kn ($F = 119.03$, p-value ***; and $F = 7.946$, p-value ***, for both stages respectively). Notwithstanding, during gonad regeneration (Figure 3P, Q, R), condition values in the Atlantic were the highest of the stocks analysed for tissue fat content, Kn, and HSI. In the Alboran (GSA 1) the highest fat content value was recorded at the spawning capability, accompanied by a high Kn, reaching similar values to the Atlantic, despite having been lower during the previous gonadal phase (i.e., developing). During the active spawn and regression, GSA 1 individuals (together with the rest of the Mediterranean stocks regarding the latter phase) surpassed in condition the Atlantic outgroup but they were below again during the regenerating phase. The Northern Spanish stock (GSA 6) presented the lowest values of Kn along the whole reproductive cycle, accompanied by low values of tissue fat content except in the developing and the spawning capability. The Northern Adriatic (GSA 17) and the Aegean (GSA 22) individuals were similar regarding the Kn values (except in the developing stage, in which the Northern Adriatic was above). Nevertheless, despite the similarities in Kn during the spawning capability and active spawning (Figure 4H, K), tissue fat content differed between these two

subregions in the regressing and regenerating phases, higher in the Aegean, but becoming lower in the developing phase (Figure 4G, J).

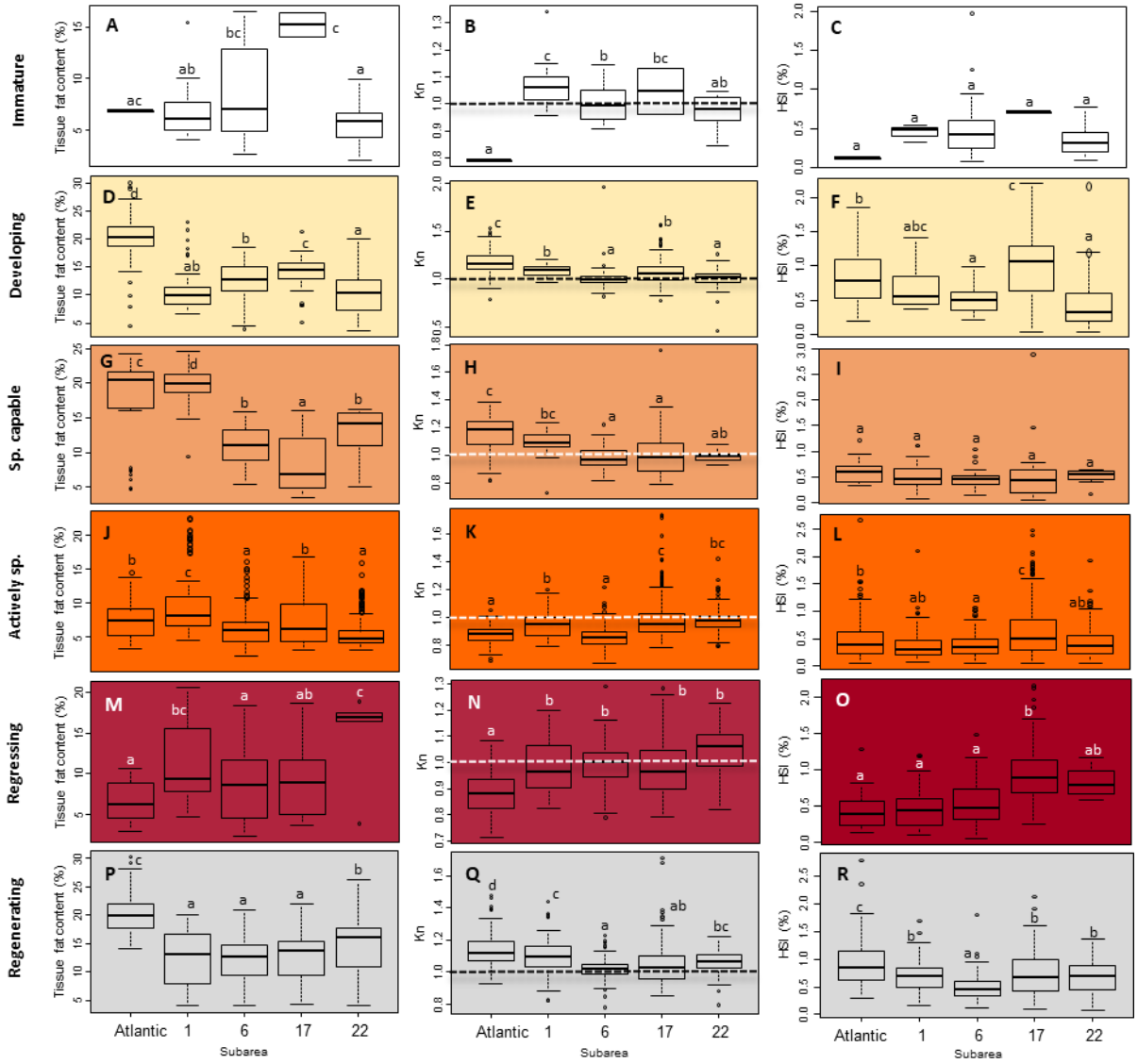


Figure 4. Tissue fat content (%), relative condition factor (Kn) and hepatosomatic index (HSI %) by subregion (GSA) along the annual reproductive cycle. Different letters on the graphs indicate significant differences among stocks.

4.3.3. Hepatosomatic index and its great divergence in the Northern Adriatic

Values of the hepatosomatic index (HSI) generally decreased right before the spawning capability, starting to grow again at the regressing phase, although some differences were appreciated (Figure 5). The outgroup (Atlantic) and Alboran (GSA 1) individuals followed the same pattern, as HSI continued increasing at the regenerating

phase (Figure 5) although only significantly differences were detected in the Atlantic to this increase from the regression to the regeneration (F value = 30.48, p-value ***). This trend was not recorded in the rest of the Mediterranean locations. Atlantic and Alboran only differed during the regenerating phase, with higher values in the Atlantic. On the other hand, sardines in the Northern Adriatic presented the highest significant values in HSI during the active spawning phase (Figure 4L), surpassing even the Atlantic outgroup and the Alboran, observed also at the subsequent regressing phase (Figure 4O). Moreover, it was recorded a light increase in HSI (despite not significant) from the spawning capability to the active spawning in this location, not occurring in the rest of the subareas (Figure 5). It is of importance to highlight these features of the livers in the Northern Adriatic because even visually their size relative to the fish was remarkable.

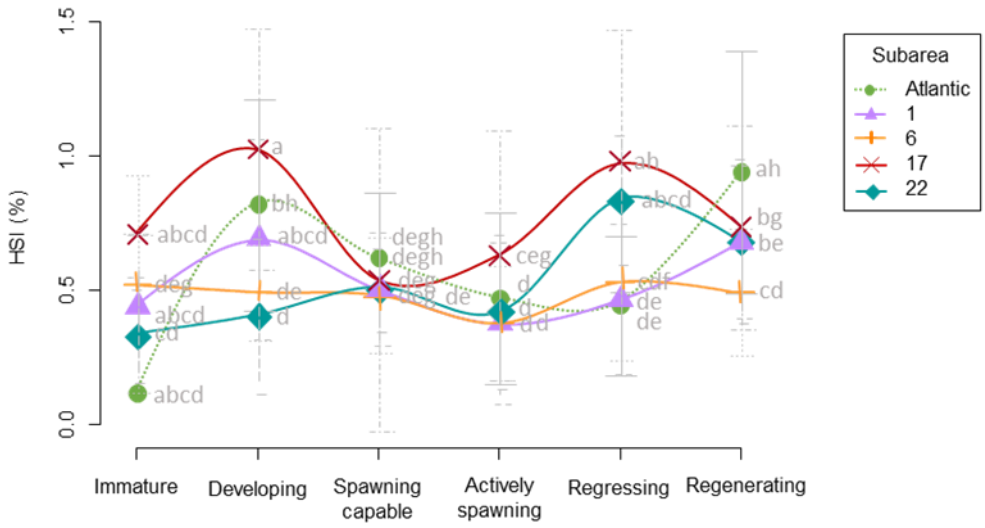


Figure 5. Hepatosomatic index (HSI) trend ± SD recorded in the five subareas of analysed by this study (GSA 1 (violet): Northern Alboran; GSA 6 (orange): Northern Spain; GSA 17 (red): Northern Adriatic; GSA 22 (turquoise): Aegean Sea)) throughout the reproductive cycle. Different letters on the graphs indicate significant differences among stocks.

4.4. Discussion

Life history traits of fish stocks are often modulated by unique environmental characteristics, which influences the phenotypical plasticity of the species and different selective and adaptive processes. Focusing on somatic condition and energy storage, fish can exhibit substantial variation in energy density (energy per unit wet weight) within a species mostly resulting from variation in the amount of stored lipids with important ecological consequences (Martin et al., 2017). Environmental factors as temperature variations may cause the exhibition of striking changes in internal biomass and energy allocation to permit the maintenance of a high level of biological activity, and the direction

and magnitude of the effect varied substantially among populations (McManus & Travis, 1998). In this context, genetic variance in energy storage has been found among fish populations (Borowsky & Kallman, 1993). It is expected, therefore, that these differences in condition influence the transfer of energy to gonadal development and, therefore, different reproductive phenology appears in species with marked reproductive seasons, as is the case of the European sardine.

In this regard, the present study shows the current spatial heterogeneity in the Mediterranean Sea in sardine reproduction and condition, reflected by both GSI and reproductive phenology, and the differences in energy storage of muscle, liver, mesentery, and the relative condition index Kn according to the annual cycle. In this line, previous global analysis of mean annual sardine body condition in the Mediterranean detected variability among locations during the period 1975-2015 (Brosset et al., 2017). Moreover, monthly local scale studies in this sea also revealed spatial variations for sardine in terms of body condition, fat content, reproduction indices and phenology, as well as of stable isotopic values (Ganias et al., 2007; Frigola-Tepe et al. 2022; Lloret-Lloret et al., 2022).

In general terms, the stock with the lowest condition parameters throughout the annual cycle was that from the Northern Spain (GSA 6), also reflected in the reproductive potential (GSI that does not exceed 3 in the actively spawning stage). Tissue fat content and Kn (only slightly surpassing the unit at the regenerating phase) were generally low, before and after the breeding season, and HSI figures were moderate through all the gonadal phases. In this area, located in the Western Mediterranean, many studies have been carried out due to the changes that have been recorded in the last decades in sardine life-history traits, and the fall in catches and biomass (e.g., Van Beveren et al., 2014; Albo-Puigserver et al., 2019). Works point at changes in the marine environment, specially at the warming pattern in the entire Western Mediterranean Sea occurred over the last 40 years at different depths and gradual salinity rise that might have substantial repercussions on the ocean currents and on the stability of water column with subsequent alterations of the nutrient supply from the deep layers to the photic zone (Quattrocchi, 2017). In addition, the effects of climate change in planktonic communities in the Western Mediterranean including the Catalan Coast are evidenced by the changes in the structure of the plankton communities and the metabolism of the ecosystem, favouring the smallest plankton (Calvo et al., 2011) and affecting the forage species as sardine. Furthermore, the disappearance of older individuals and truncation of the age-structure have been observed in the area (Albo-Puigserver et al., 2021), limiting the ability to deal with environmental events and affecting the quantity and quality of sardine spawn (Brosset et al., 2016).

On the other hand, Northern Adriatic stock (GSA 17) reflects a trend whose condition seems to be generally above the other stocks analysed during the actively reproductive period. Thus, we conjecture that this is due to the fact that sardines in this area (Gulf of Trieste) have accessible feeding resources all year round in the pelagic ecosystem (Conversi et al., 2009) (Figure S4.2 B), mainly motivated by its geographical location and its semi-enclosed basin (Brosset et al., 2017), in addition to the deposit of nutrients by rivers, being even more eutrophic at late winter-spring when sufficient inorganic nutrients are available to sustain the main diatom bloom of the year (Umani et al., 2012), and coinciding with sardines' reproductive season. In fact, the typical phytoplankton seasonal succession in Northern Adriatic displays the following pattern: a late winter-early spring diatom bloom, followed by a nanophytoplankton bloom in late spring-early summer, a cyanobacteria peak in late summer-early autumn, and a second relative diatom maximum, usually in November (Cibic et al., 2018). Nevertheless, it has to be considered that in Gulf of Trieste, sardines fed mainly on copepods (56 %) and phytoplankton never exceeds 10 % of the prey (Borme et al., 2022), although the large primary production promotes the abundance of this zooplanktonic species. Furthermore, HSI reflected the highest values and a different trend compared to the rest of the stocks. In the present study, we surprisingly observed great livers that started to grow before and during the active reproduction, overlapping with this greater availability of food in the mid-autumn (spawning capable and active spawners) and winter-early spring season (active spawners). In the study of Ganias et al. (2007) performed in two highly oligotrophic seas (central Aegean and Ionian), however, seasonality of spawning did not match variations of HSI, although it was consistently higher in reproductively active females during the whole spawning period. In this sense, everything seems to indicate that liver is not the main lipid storage compartment in sardine (Bandarra et al., 2018) but has some relation to fish condition and feeding activity, playing a role in nutrient transfer between the different organs. The accumulation of fat around the gut (mesenteric fat) and within the muscle are the main lipid storage compartments to be allocated to reproduction (Nunes et al., 2011). This hypothesis could be reinforced by the fact that in our study it was observed that in stocks in which there is little availability of resources throughout the year, in the Aegean Sea, there is higher HSI during the regressing-regenerating phases, coinciding with a peak in production in this sea, and in Northern Spain, a flat trend in HSI is present during the whole annual cycle. Besides, in this area of the Northern Adriatic the greatest temperature difference between the winter and summer months was recorded compared to other subareas, which also can alter the way of storing and allocating energy, as previously discussed, as well as trigger the reproductive period, reaching the spawning peak in autumn, the earliest of the analysed. Taking into account the relatively high condition indices of the Adriatic stock in the reproductive period, we can

conclude that the classification of the European sardine as a capital breeder would be conditioned by the population under study. Geographical location can determine changes in feeding behaviour of adults of sardines (Rumolo et al., 2016) and, as reported in the Adriatic, in winter it is detected the lowest percentage of empty stomachs (Zorica et al., 2016), since the feeding intensity of sardine is higher when productivity is high (Nikolioudakis et al., 2011). Therefore, we propose that the storage period and the mobilization of energy for reproduction in the Northern Adriatic is not as marked as it could be in other stocks. Thereby, our findings imply that besides capitalized energy, sardine also uses current income for supporting reproduction (Ganias, 2009), and that the degree of dependence of reproduction on immediate reserves is closely linked to the availability of resources. These traits may be the result of local adaptation processes of the Adriatic population, which seems to differ genetically from other Mediterranean stocks (Caballero-Huertas et al., 2022a).

The Aegean stock (GSA 22) presented low values of somatic condition from the developing period to the actively spawning, but the recovery at the regressing phase was the largest of all the stocks analysed in this study, reflected by the tissue fat content and Kn values. In the regenerating phase, however, the Atlantic stock exceeded these values, although GSA 22's individuals show the best condition parameters in the Mediterranean in this phase. In this aspect, a peak in primary productivity is recorded in the area during the summer, in which a large fraction of individuals in a state of regression was captured, so it is expected that these individuals are feeding intensely at this time in the area and, therefore, present better parameters, to which is added a high HSI value similar to that of the Adriatic (GSA 17). The rising temperatures since spring, turning GSA 22 into the subarea with the warmest summers and autumns analysed, do not seem to affect the condition of the sardine in that time window. In fact, sardines at the Thermaikos Gulf are further selective for warm waters (Giannoulaki et al., 2005), which shows the great plasticity of this species throughout its distribution range, although it may be probably associated with the greater availability of resources during this time period.

Northern Alboran sardines followed a similar reproductive seasonality than the Atlantic individuals, coinciding with its closeness in space to this area. However, an earlier maturation of the gonads was recorded in summer in the Atlantic, although no active spawners were present in autumn, what was observed in Alboran in this season. As proposed in a recent work, we hypothesize that Atlantic individuals enter in Mediterranean waters when they are able to spawn and triggered by a drop in temperature in the Alboran coast, to which could favour the establishment of the important sardine nursery grounds along the northern coast of the Alboran Sea, Málaga, and Almería Bay (Caballero-Huertas et

al., 2022c), occurring the overlapping of stocks in GSA 1. Despite their similarities regarding the reproductive phenology, we could observe differences regarding the energy storage and condition along the reproductive cycle. During the spawning capability, the relative condition (Kn) was similar in both stocks, although the energy stored in the muscles was slightly higher in the Alboran. From this moment on in the reproductive cycle, a better condition was observed in the Alboran zone in terms of both indices, although in the state of regeneration, the Atlantic individuals were able to recover better and more quickly. In addition, a common pattern of the HSI was share, with no significant differences between stocks except in the regeneration state, higher in the Atlantic. This relatively low condition during the actively spawning phase in the Atlantic is possibly linked to a highest destination of resources to reproduction (one of the highest GSI values recorded in this study, of 5.23 ± 2.46, Table S4.3). Moreover, the faster recovering during the regeneration may be due to the early energy acquisition and reserve recruitment facilitated in an area with a continue nutrient input (Caballero-Huertas et al., 2022c). These reasons could be key to understand why sardine abundance is generally lower in the Mediterranean than in the Atlantic waters (Silva et al., 2008). The spatio-temporal analysis of sardine health status for different Mediterranean areas (Brosset et al., 2017) revealed a drastic decrease in the condition and maximum size of the specimens captured between 1975 and 2015 in most areas analysed, with the exception of the Northern Alboran Sea (GSA 1), where the condition notably improved from 2010 on, although Albo-Puigserver et al. (2021) began to see signs of decline. Our results showed that the Alboran is the Mediterranean subarea in which the highest condition values are generally recorded throughout the annual cycle. After this fact, it could be the potential genetic variability and plasticity induced by the overlapping of Atlantic and Alboran individuals in this locality (Albo-Puigserver et al., 2021; Caballero-Huertas et al., 2022c). Moreover, the temperatures in this Mediterranean subarea are more favourable for this species with cold-temperate affinity, with less marked seasonal variations than in the other localities.

Due to the importance of condition in the health status of sardine stocks and its direct influence on recruitment, the monitoring of this species from the reproductive framework (related to its annual cycle) is essential as a way to contextualize its status from a biologically relevant perspective. Moreover, the different reproductive phenology among stocks makes us suggest that the closed periods during the reproductive season must be established according to the features of each stock. In several subareas, closed seasons do not completely overlap with the spawning capable-actively spawning phases, which can be problematic for the recruitment and the health of the stocks. This occurs, for example, in the

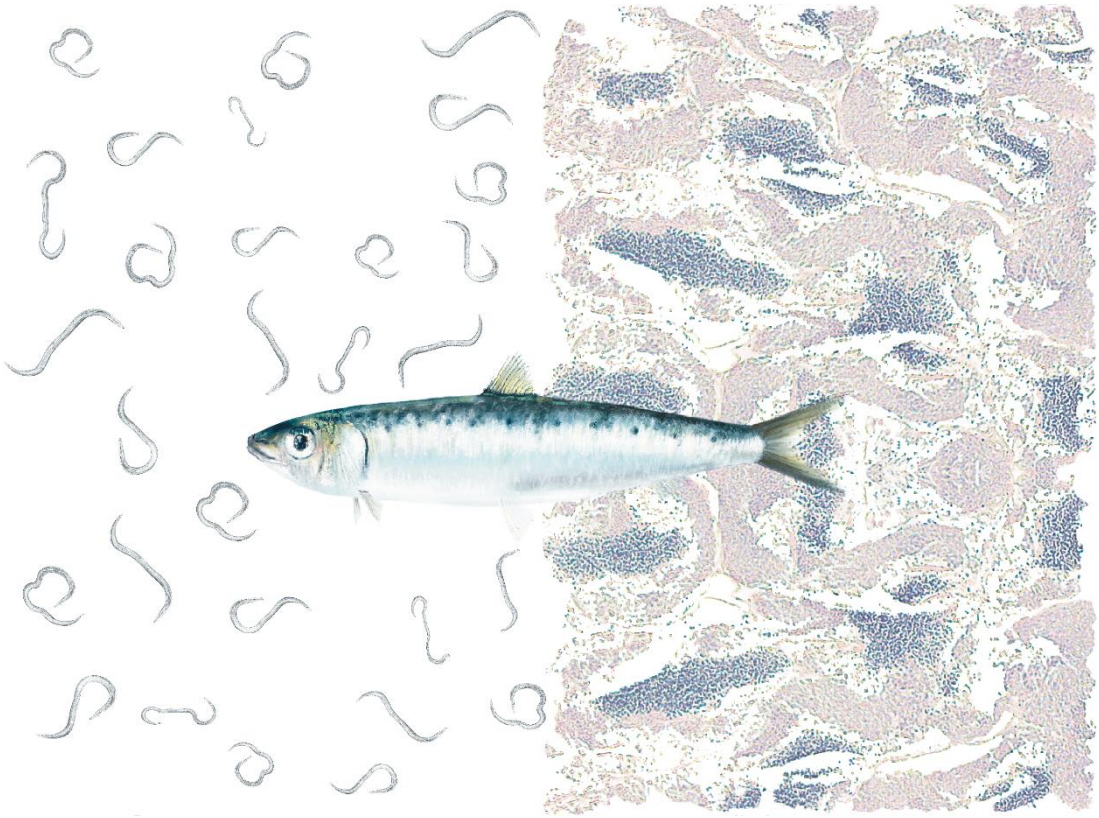
Northern Adriatic (GSA 17), as the closed season in August (Farrugio & Soldo, 2014) does not protect the spawning peak of sardine in the area, from November to February.

In the same way, this study allows us to observe the heterogeneity in stocks of a same species, which causes that management measures should be established respecting the particularities of each stock, but taking into account the potential connectivity of some of them (Atlantic stocks of the Gulf of Cadiz-South of Portugal and Alboran). In this regard, our proposal is to assess and manage the southern area of Portugal - south-western Spain (Southern European Stock, ICES Subareas 27.8.c and 27.9.a, fished by Spain and Portugal (ICES, 2017)) as a co-shared stock with the GFCM, considering combined GSAs 1, 2 and 3. This would even be potentially useful for other species in which connectivity may play a fundamental role, as the European hake and the blackspot seabream (García-Lafuente et al., 2021).

Due to the dynamism of environmental conditions accentuated by global change, especially in the Mediterranean region, the recurrence of this type of analysis with environmental discussion is essential. In fact, life history traits that rapidly respond to environmental changes, such as body condition, are good indicators to foresee future population declines (Lloret et al., 2012; Albo-Puigserver et al., 2021). Furthermore, it is important to incorporate new stocks to understand the status of the species in a global way. Future studies should evaluate stocks in Northern Sea, Mediterranean and Atlantic African Coast, the most eastern Mediterranean Sea, etc. to continue building knowledge on the status of this important fishery resource.

SECTION III

LINKING CONDITION TO PARASITISM AND PATHOLOGIES IN EUROPEAN SARDINE



Chapter 5

Ascaridoid parasites in European sardine throughout the annual cycle: variability in parasitic load according to host stock features

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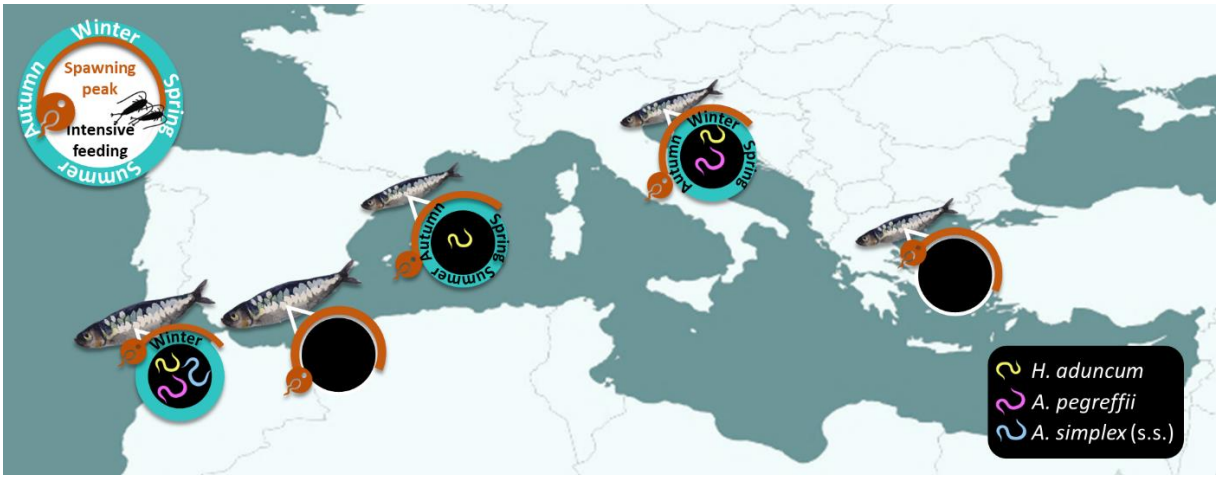

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Abstract

In recent years, a drop in the condition of the European sardine has been observed. Although several causes have been attributed to this issue, as overfishing and climate change, little is known about the link between ascaridoid nematode parasitisation and fish status. In this study, sardines were obtained from four fishing grounds along the Mediterranean (Alboran, Northern Spain, Northern Adriatic, and Aegean), and one location in the Atlantic Ocean (Southern Portugal). After analysing individual fish body condition (by direct tissue fat content measurements and condition indices), and reproductive status (by a detailed gonadal examination) throughout the entire annual cycle, ascaridoids were recognised by combining naked eye and UV-press method along flesh, viscera, and gonads. Afterwards, sequence analysis of the rDNA internal transcribed spacers region (ITS) and the mtDNA cox2 gene were used to identify and characterise the different species of ascaridoids from the fish host in the localities throughout the seasons. The main species found along different areas was *Hysterothylacium aduncum*, present in the Northern Adriatic (prevalence of 7.6%, mean intensity 1.700), the Atlantic (7.5%, 3.889), and the Northern Spain (3.9%, 1.600). Moreover, few individuals of *Anisakis simplex* (s.s.) and *A. pegreffii* were observed in the Atlantic (1.7 % and 0.8%, respectively), and the latter species was also found in the Adriatic stock (0.8%). All ascaridoid specimens were found in viscera. Obtained results seem to indicate that in stocks with medium sizes, small variations in length are related to parasite intensity. This study highlights the importance of seasonal parasitological analyses at stock level and, especially, in capital breeders, as relationships between condition and reproduction parameters and parasitism are conditioned by seasonality.

Keywords: *Anisakis* • Capital breeder • Condition • *Hysterothylacium aduncum* • Pelagic • *Sardina pilchardus*

5.1. Introduction

European sardine (*Sardina pilchardus* (Walbaum, 1792)) is a small pelagic fish from the Clupeidae family with cold-temperate water affinity, living within a depth range of 10 - 100 m (Renzi et al., 2019). It plays an important role in the ecosystem as a filter feeder (Cury et al., 2000; Van Beveren et al., 2014) that feeds mainly on planktonic crustaceans, appendicularians, diatoms and other organisms (Costalago & Palomera, 2014), contributing to the energy transfer to higher trophic levels. From the point of view of human nutrition, sardine is rich in long-chain polyunsaturated fatty acids (PUFA), essential for human development and the prevention of many health disorders, as well as easy digestible proteins which contain all essential amino acids necessary for healthy human diets, minerals, and vitamins (Šimat et al., 2020).

It is found throughout the northeast of the Atlantic Ocean, from the North Sea to Mauritania and Senegal, the Sea of Marmara, the Black Sea, and the Mediterranean Sea (Parrish et al., 1989). Specifically, in the latter most stocks have exhibited declining trends in terms of abundance (Tugores et al., 2011; FAO, 2020; Fernandes et al., 2017), remaining low in the present day (FAO, 2020). In addition to the problems that could be reflected in the food chain due to its relevance as a foraging species, the economic consequences are obvious since together with anchovy, sardine traditionally provides the largest catches in this area (Lleonart & Maynou, 2003).

This decline in production is linked to a decrease in somatic condition, length at age, and size at first maturity (Brosset et al., 2017; Albo-Puigserver et al., 2018; Ramírez et al., 2021) and ultimately, in fish health status. Certain hypotheses have been confirmed about the environmental agents that are having the mentioned effects in the resource, such as fishing pressure, the increase in temperature as a result of the global warming, and their combination (Ramírez et al., 2018; Fernández-Corredor et al., 2021). Furthermore, biological factors such as food availability or parasites have been proposed as potential agents behind the drop in condition in fish. Studies have pointed at the productivity and composition of the zooplankton to be related to sardine status (El Mghazli et al., 2020; Mercado et al., 2007). However, a gap of information is found regarding the relationship between nematode parasitism and this small pelagic. Piscivorous species such as hake and cod are usually more heavily infected by ascaridoid nematodes compared to strict plankton feeders such as sardine, since abundance and spatial distribution of larvae seems largely to depend on fish host species and their respective feeding behaviour (Mattiucci et al., 2018). Thereby, the principal focus on ascaridoid parasitism has been mainly on the former, being less studied in planktivorous species as sardine, which feeds principally on zooplankton

(mainly copepods, but also cladocerans, euphausids, crustacean larvae or anchovy eggs, among others), although phytoplankton is also consumed (Palomera et al., 2007; Rello et al., 2008). Nevertheless, as parasitism may affect host physiology, morphology, reproduction and behaviour (Timi & Poulin, 2020), it must be considered as a potentially determining factor in the state of health and population dynamics of the stocks, and especially the context of vulnerability in which sardines are found. Similarly, the inverse relationship between parasitism and sardine condition has been suggested, as healthier fish stocks could be considered more resistant to parasite infections (Pennino et al., 2020a; Frigola-Tepe et al., 2022). Furthermore, the current state of the habitats is being altered by environmental pressures derived from global change, which could also be conditioning and altering the dynamics and life cycles of ascaridoid nematodes. In fact, warming of coastal waters potentially result in a higher number of pelagic fish species that follow warmer currents northwards, which would increase *Anisakis* spp. infection of fish, added to a general shift in host ranges and the introduction of pathogens into formerly uninfected regions (Klimpel & Palm, 2011).

In this way, it is of interest to analyse from an ecological perspective and taking into account the host (i.e., sardine) health parameters to evaluate the state of the fishing resource, since most studies that assess the parasitisation of this species by ascaridoids and, especially, by *Anisakis* spp., are carried out from the point of view of zoonoses and food safety (see, for example, Santos et al., 2006; Piras et al., 2014; Serracca et al., 2014; Bao et al., 2020). This occurs because the intake of the sibling species *Anisakis simplex* sensu stricto (s.s.) and *A. pegreffii* of the *A. simplex* sensu lato (s.l.) complex are the main causative agents of digestive or allergic symptoms to the human consumer if the fish that carried the larval nematode was not subjected to high temperatures or frozen previously to the ingestion (Villafruela-Cives et al., 2010; Roca-Geronès et al., 2021). In fact, human anisakiasis by European sardine consumption (either fresh, marinated or canned) has been reported over time and across countries (e.g., in Spain (López-Serrano et al., 2000; Molina-Fernández et al., 2015), Italy (Guardone et al., 2018), etc.). Nevertheless, there are other ascaridoid species that are not prone to cause these reactions in human consumers but that produce negative impacts on larvae and adult fish, as *Hysterothylacium aduncum* (Balbuena et al., 2000; Dallarés et al., 2016).

That is why this study intends to combine the mere description of the parasitic load of ascaridoid nematodes in sardines with ecological information on this small pelagic, since in this way we will be able to address both the immediate interest in terms of safety for the human consumption as well as exploring the fish resource status by linking this parasitism with the reproductive and energetic characteristics of sardine along different areas of its

distribution. Within this context, the aims of this study have been (1) to characterise the nematode parasites in the European sardine along its distribution (stocks from the Atlantic and Mediterranean) by investigating the features of the infection and its occurrence throughout the seasons and (2) to relate these aspects of the infection with hosts' reproductive cycle and body condition, as well as with further biological traits and environmental information.

5.2. Materials and methods

5.2.1. Body condition and reproduction analyses

Specimens of *Sardina pilchardus* (N = 760) were collected seasonally (seasons defined as winter: January, February, March; spring: April, May, June; summer: July, August, September; autumn: October, November, December) from the end of 2019 to 2021 along the Southern Portugal-Gulf of Cádiz coast (Northeast Atlantic Ocean, Portuguese Waters - East (FAO fishing area division 27.9.a)), the coast of Málaga, bathed by the Alboran Sea (Mediterranean Sea, GFCM – GSA 1), Catalan Coast (Balearic Sea, GFCM – GSA 6), Trieste (Adriatic Sea, GFCM – GSA 17), and Thessaloniki (Aegean Sea, GFCM – GSA 22) by commercial fisheries (Figure 1). Immediately after the purchase, samples were frozen at –20 °C, which has been demonstrated that it has no significant effect on the studied parameters (Brosset et al., 2015a). Each sardine was measured (total length, L_T ± 0.1 cm) and weighed (total body weight, W_T ± 0.01 g); eviscerated body weight, W_E ± 0.01 g). Gonad (W_G ± 0.0001 g) and liver (W_L ± 0.0001 g) were also weighted. Body condition was obtained by the calculation of the relative condition index (Kn) (Le Cren, 1951) ($Kn = W_E / \alpha \cdot L_T^{\beta}$), where W_E is eviscerated weight, L_T is total length, and α and β are constants obtained by the regression line of the logarithms of length and mass from the samples per location. The gonadosomatic ($GSI = 100 \cdot [W_G / W_E]$) and hepatosomatic ($HSI = 100 \cdot [W_L / W_E]$) indexes were calculated. The sex of each specimen was determined macroscopically, and gonads were classified according to the criteria of Brown-Peterson et al. (2011) (i.e., immature, developing, spawning capable, actively spawning, regressing, and regenerating). Regarding the lipidic and energetic body condition, tissue fat content (%) was estimated by the average of both sides using a fish fatmeter (Distell Model FM 992, SARDINE-2 calibration), and a visual scale for fat mesenteric reserves with seven levels, in which level 1 represents

invisible or thin and indistinct fat lines, and level 7 consists in fat line lobes and fish fundulus well-covered with fat (Van Der Lingen & Hutchings, 2005).

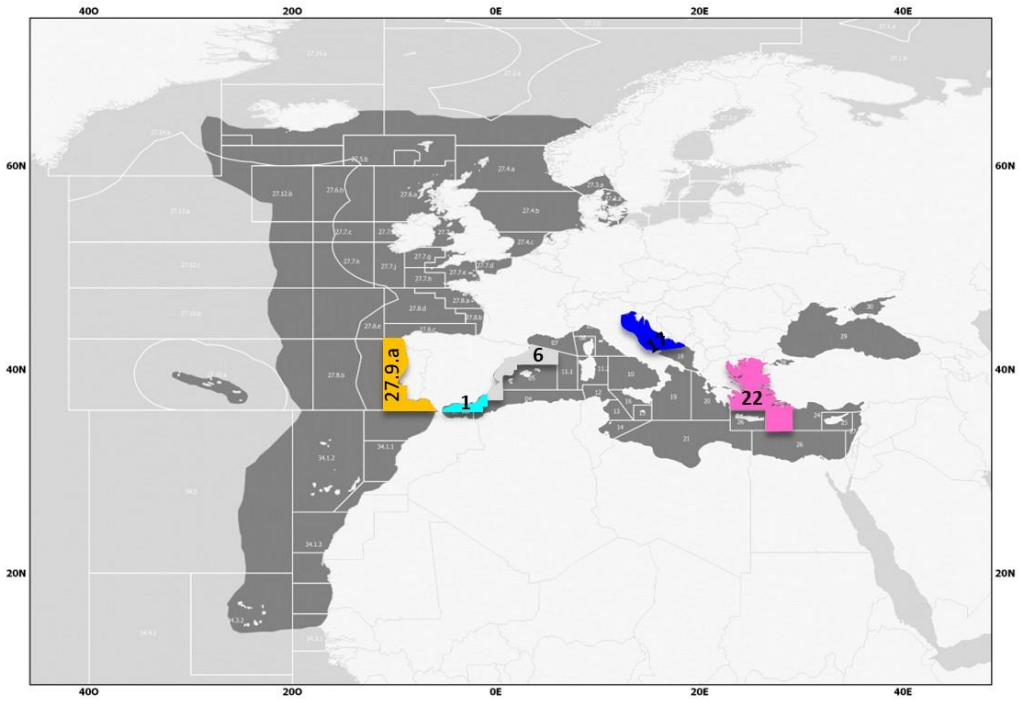

Figure 1. Map of the European sardine (*Sardina pilchardus*) stocks sampled along its distribution (in dark grey) by subareas (FAO divisions in the Atlantic and GFCM - GSAs (into FAO Major Fishing Area 37 in the Mediterranean)). Orange: FAO Division 27.9.a Portuguese Waters - East; blue: GSA 1 Alboran; light grey: GSA 6 Northern Spain; electric blue: GSA 17 Northern Adriatic; pink: GSA 22 Aegean.

5.2.2. Parasitological analysis

5.2.2.1. Parasite inspection method

Sardine gills were analysed by plain visual inspection. The stomach and intestine were opened to expose their content and the nematode detection was carefully performed using forceps and a lamp by naked eye. After, the whole digestive tract including the pyloric caeca, liver and gonads were placed into a transparent 1-3 mm plastic bag next to the flesh cut into butterfly fillets. Bags were then pressed under hydraulic pump and stored overnight at −20 °C for further inspection by UV-press method. The method is based on the fluorescence of frozen ascaridoid larvae, which allows the visual inspection of flattened/pressed and subsequently deep-frozen fish fillets or viscera under UV-light exposure at 366 nm in a darkened room (Cipriani et al., 2018; Levsen et al., 2018; Mattiucci

et al., 2018). Afterwards, all parasites were counted, and their anatomical location was reported.

5.2.2.2. Ascaridoid identification by direct sequencing

Firstly, a visual identification of the nematode to genus level based on morphology was carried out following Moravec (1994) and Berland (1961) criteria. Then, total DNA from each specimen was extracted using a Quick-gDNA MiniPrep (Zymo Research Corp, CA, USA), following the manufacturer's protocol. The ITS region of the rDNA including the first internal transcribed spacer (ITS-1), the 5.8S gene, the second transcribed spacer (ITS-2), and ~70 nucleotides of the 28S gene, was amplified using the primers NC5 (forward, 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse, 5′-TTAGTTTCTTTTCCTCCGCT-3′) as reported in Zhu et al. (2000). PCRs were carried out following the protocol reported in Palomba et al. (2021). Additionally, the mitochondrial cytochrome *c* oxidase subunit II (mtDNA *cox2*) gene locus was amplified using the primers 211F (forward, 5′-TTTTCTAGTTATATAGATTGRTTYAT-3′) and 210R (reverse, 5′-CACCAACTCTTAAAATTATC-3′). PCRs were carried out following the protocol reported in Mattiucci et al. (2014). The successful PCR products were purified, and Sanger sequenced by BioFab Research (Italy, Rome). The sequences obtained were analysed and edited by using Chromas Pro 1.34 and MEGA X v. 11 (Kumar et al., 2018). Sequence identity was checked using the Nucleotide Basic Local Alignment Search Tool (BLASTn) (Morgulis et al., 2008).

5.2.3. Statistical analysis

5.2.3.1. Analysis of the infection data

Quantitative Parasitology 3.0 software (Reiczigel et al., 2019) was applied to calculate the infection levels of *Anisakis* spp. and *Hysterothylacium* spp. larvae by sardine sampling area. General prevalence (P, %) with confidence limits (Clopper–Pearson interval, confidence level of 95 %), mean intensity (mI) (Bootstrap BCa with confidence level of 95 %, 2000 replications), and mean abundance (mA) (Bootstrap BCa with confidence level of 95 %, 2000 replications) were obtained. The significance of statistical differences in prevalence, assessed by the Fisher's exact test, and mean intensity and abundance of larvae,

analysed by bootstrap one-way ANOVA with 1000 replications, were checked by location/sardine stock. Differences were considered significant when p-value < 0.05*.

5.2.3.2. Analysis of host-parasite relationships

Using R software version 3.5.1. (R Development Core Team, 2018), Shapiro-Wilk test was applied to test the assumption of normality and Levene's test was executed to check the homogeneity of variances (Zar, 1996) in all parameters calculated. If both assumptions were met in the analysis carried out per stock comparing infected and non-infected individuals, independent samples t-test was performed. Conversely, when only homoscedasticity assumption was violated, data was analysed with Welch's t-test. For those parameters in which normal distribution was lacking but homoscedasticity was present, Kruskal-Wallis analysis of variance was applied. Chi-square test of independence was applied when testing the dependence of two categorical variables (e.g., parasite presence/absence vs. sex or mesenteric fat). The Spearman's rank non-parametric correlation test was used to explore the relationship between the number of nematodes and the sardine parameters (i.e., total length (cm), Kn, GSI (%), and HSI (%)). Statistically significant differences were considered if p-value < 0.05*.

5.3. Results

5.3.1. Detection and identification of Hysterothylacium spp. and Anisakis spp. larvae

A total of 69 and 5 larval nematodes were assigned morphologically to the genus *Hysterothylacium* (Raphidascaridae family) and *Anisakis* spp. (Anisakidae family), respectively. Differences in the light reflected by the ascaridoids were appreciated under UV light: *Anisakis* spp. had an intense violet hue (Figure 2A), while *Hysterothylacium* spp. emitted a weak yellowish light (Figure 2B). All larvae were located in the visceral cavity of the examined sardines, mainly placed on the pyloric caeca. No parasitic individual was observed in flesh for any of the locations.

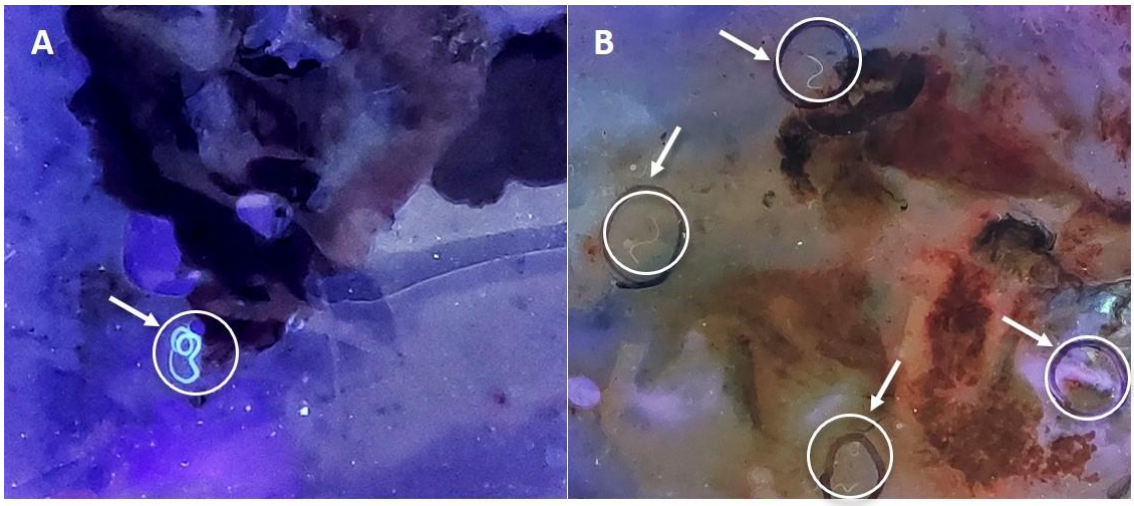

Figure 2. Ascaridoid nematodes observed under the UV-press method. A specimen of *Anisakis simplex* (s.s.) (**A**) and four individuals of *Hysterothylacium aduncum* (**B**) found in sardines from the Atlantic stock (FAO Division 27.9. a Portuguese Waters – East).

According to the sequences obtained at the ITS region of the rDNA (800 bp), 2, 3 and 38 larvae showed 100 % identity with the sequences of *A. pegreffii* (accession number (a.n.) JX535520 [Mattiucci et al., 2014]), *A. simplex* (s.s.) (a.n. JX535521 [Mattiucci et al., 2014]) and *H. aduncum* (a.n. JQ934878 [Vardić Smrzlić et al., 2012]), previously deposited in GenBank. The mtDNA cox2 gene locus (580 bp), also identified the same larvae of *A. pegreffii*, *A. simplex* (s.s.) and *H. aduncum* showing > 99% identity with sequences previously deposited in GenBank (*A. pegreffii*, a.n. JQ900761 [Mattiucci et al., 2013]; *A. simplex* (s.s.), a.n. DQ116426 [Valentini et al., 2006]; and *H. aduncum*, a.n. OK338702 [Karami et al., 2022]). Sequences obtained were deposited in GenBank under accession numbers: OP975692-97 (ITS) and OP985157-62 (*cox2*).

5.3.2. Levels of ascaridoid infection in European sardine

Significant geographical differences in general prevalence of *H. aduncum* were observed among individuals (two-sided p-value: < 0.0001***). It was present in the Northern Adriatic (prevalence of 7.6 %), the Atlantic (7.5 %) and the Northern Spain (3.9 %), and absent in the Alboran and Aegean Sea (Table 1). Prevalence and mean abundance for *H. aduncum* were significantly different by areas (two-sided p-value: < 0.0001***; Bootstrap one-way ANOVA with 1000 replications resulted in p-value = 0.037*, respectively). Mean intensity did not present significant differences among locations (Bootstrap one-way ANOVA with 1000 replications resulted in p-value = 0.333) (Table 1).

Table 1. Data on number of infected European sardines, number of nematodes recovered, epizootiological parameters (prevalence (P), mean intensity ± standard deviation, mean abundance ± standard deviation) by subareas from the Atlantic (Atl) or Mediterranean (Med).

Subarea	Infection parameters	Ascaridoid		
		Hysterothylacium aduncum	*Anisakis pegreffii*	*Anisakis simplex* (s.s.)
South Portugal (FAO Division 27.9.a) Atl $N_{analysed\ fish}$ = 120	N infected fish	9	1	2
	N larvae	35	1	3
	P (%)	7.5	0.8	1.7
	mI (± SD)	3.889 ± 5.278	1.000 ± NA	1.500 ± 0.707
	mA (± SD)	0.292 ± 1.712	0.008 ± 0.091	0.025 ± 0.203
Alboran (GSA 1) Med $N_{analysed\ fish}$ = 119	N infected fish	0	0	0
	N larvae	0	0	0
Northern Spain (GSA 6) Med $N_{analysed\ fish}$ = 259	N infected fish	10	0	0
	N larvae	16	0	0
	P (%)	3.9	0	0
	mI (± SD)	1.600 ± 0.843	0	0
	mA (± SD)	0.062 ± 0.347	0	0
Northern Adriatic Sea (GSA 17) Med $N_{analysed\ fish}$ = 131	N infected fish	10	1	0
	N larvae	18	1	0
	P (%)	7.6	0.8	0
	mI (± SD)	1.700 ± 0.823	1.000 ± NA	0
	mA (± SD)	0.130 ± 0.502	0.008 ± 0.087	0
Aegean Sea (GSA 22) Med $N_{analysed\ fish}$ = 131	N infected fish	0	0	0
	N larvae	0	0	0

Anisakis pegreffii was only observed in the Atlantic and the Northern Adriatic (prevalence of 0.8 % in both stocks). Significant differences among areas were found neither for prevalence (Two-sided p-value: 0.3748) nor for mean abundance (Boostrap one-way ANOVA with 1000 replications resulted in p-value = 0.619). For mean intensities, within-

group variability could not be assessed, therefore reliable comparison of means could not be made.

Anisakis simplex (s.s.) was only found in the Atlantic (prevalence of 1.7 % in the area). Significant differences among localities were found for prevalence (two-sided p-value: 0.0491*). Differences in mean abundance were not significant (Boostrap one-way ANOVA with 1000 replications resulted in p-value = 0.168). For mean intensities, within-group variability could not be assessed, therefore reliable comparison of means could not be made.

No ascaridoid was observed in the Alboran or the Aegean.

Significant differences among seasons were found in the parasitised locations. In the Atlantic stock, all identified *H. aduncum* were found in winter (a prevalence of 30.0 % in the sardines analysed in this season, two-sided p-value: < 0.0001*). All *Anisakis* spp. in this area were also observed in winter. However, due to the low prevalence, no significant differences among seasons were obtained. In Northern Spain, significant higher prevalence of *H. aduncum* (two-sided p-value: 0.0311) was recorded in spring (9.3 %), followed by summer (3.1 %) and autumn (1.7 %). In this location, no *H. aduncum* was observed in winter. In the Northern Adriatic, higher prevalence of *H. aduncum* was recorded in autumn (15.0 %), although no significant (two-sided p-value: 0.1279), followed by winter and spring (6.7 % for both seasons). However, mean intensity in autumn was significantly higher (2.167 ± 0.753 in autumn, and 1.000 ± 0.000 in winter and spring) (Boostrap one-way ANOVA with 1000 replications resulted in p-value = 0.046*). The only specimen of *A. pegreffii* found in the Adriatic was seen in spring.

5.3.3. Linking sardine condition and reproduction with nematode parasitisation by fishing ground

After the analysis of sardines' condition parameters (Table 2), significant opposite patterns between reproductive (i.e., GSI) and body fat (i.e., tissue fat content and mesenteric) indices were observed in the global analysis of the annual data (Figure 3). Moreover, Kn was negatively related to GSI in all the stocks except in the Northern Adriatic. Kn was also significantly and positively linked to body fat parameters (Figure 3A). It was registered a general increasing trend from winter to summer regarding sardine body reserves, while the GSI started to decrease from winter on, recovering about summer and autumn months, although there were peculiarities linked to the stock (Figure 3B).

Table 2. Summary of the sampled individuals of European sardine (*Sardina pilchardus*). N: number of total individuals and the identified by sex (F, female or M, male); Kn: Le Crens's index; GSI: gonadosomatic index; HSI: hepatosomatic index; N_{coll}: collected ascaridoid larvae; N_{id}: identified larvae.

		Sardina pilchardus								**Ascaridoid nematodes**	
Area	Season	N		Total length ± SD (cm)	Kn	Tissue fat content ± SD (%)	Mesenteric fat (median) (#)	GSI ± SD (%)	HSI ± SD (%)	N_{coll}	N_{id}
		F	M								
South Portugal (FAO Division 27.9.a) Atl	Winter Feb, Mar	30		18.38 ± 0.86 (16.60 - 20.10)	0.83 ± 0.06 (0.70 - 1.01)	8.11 ± 2.38 (4.50 - 12.60)	1 (1 - 2)	5.98 ± 3.13 (0.27 - 13.14)	0.52 ± 0.37 (0.05 - 1.40)	39	34
		16	14								
	Spring Jun	30		17.96 ± 0.63 (16.90 - 20.10)	1.00 ± 0.05 (0.92 - 1.12)	20.35 ± 4.07 (14.25 - 27.90)	7 (6 - 7)	0.62 ± 0.24 (0.17 - 1.25)	0.87 ± 0.46 (0.28 - 2.36)	0	0
		23	7								
	Summer Sep	30		17.07 ± 1.56 (14.40 - 19.80)	1.08 ± 0.10 (0.85 - 1.28)	20.81 ± 2.71 (12.20 - 27.10)	7 (6 - 7)	0.85 ± 0.37 (0.22 - 2.01)	0.71 ± 0.35 (0.24 - 1.74)	0	0
		18	12								
	Autumn Oct	30		18.39 ± 0.56 (17.40 - 19.70)	1.13 ± 0.07 (1.05 - 1.34)	19.06 ± 1.61 (15.65 - 22.85)	7 (6 - 7)	1.89 ± 1.40 (0.52 - 5.77)	-	0	0
		12	18								
Alboran (GSA 1) Med	Winter Feb	20		19.24 ± 0.91 (15.90 - 20.40)	0.91 ± 0.04 (0.84 - 0.99)	8.58 ± 1.88 (4.75 - 11.25)	1 (1 - 2)	9.26 ± 3.32 (2.50 - 14.17)	0.24 ± 0.10 (0.08 - 0.48)	0	0
		7	13								
	Spring Apr, Jun	30		16.53 ± 3.57 (10.90 - 20.20)	0.94 ± 0.06 (0.83 - 1.06)	6.72 ± 1.90 (3.90 - 12.40)	2 (1 - 3)	1.86 ± 1.59 (0.15 - 6.00)	0.39 ± 0.25 (0.07 - 0.97)	0	0
		11	9								
	Summer Aug	39		16.18 ± 1.12 (10.80 - 17.50)	1.10 ± 0.06 (0.90 - 1.19)	16.74 ± 2.51 (6.95 - 20.60)	7 (1 - 7)	0.24 ± 0.19 (0.060 - 0.86)	0.81 ± 0.28 (0.34 - 1.69)	0	0
		17	21								
	Autumn Oct	30		19.53 ± 0.60	1.15 ± 0.05	20.29 ± 2.42	6 (5 - 7)	4.56 ± 1.57	0.51 ± 0.21	0	0
		12	18								

			(18.40 - 20.50)	(1.02 - 1.25)	(13.00 - 24.55)		(1.62 - 8.02)	(0.23 - 1.12)		
Northern Spain (GSA 6) Med	Winter Feb, Mar	60 (37 / 23)	13.41 ± 1.35 (10.80 - 16.60)	0.92 ± 0.08 (0.82 - 1.31)	5.68 ± 2.13 (2.15 - 11.15)	1 (1 - 4)	2.59 ± 1.89 (0.24 - 6.81)	0.50 ± 0.30 (0.06 - 1.49)	0	0
	Spring May, Jun	75 (47 / 26)	13.29 ± 1.09 (9.50 - 16.00)	1.02 ± 0.07 (0.61 - 1.20)	9.70 ± 3.48 (3.50 - 18.40)	4 (1 - 7)	0.32 ± 0.19 (0.06 - 0.97)	0.62 ± 0.34 (0.19 - 1.81)	13	1
	Summer Jul, Aug	64 (41 / 22)	13.57 ± 0.93 (10.90 - 15.10)	1.06 ± 0.07 (0.89 - 1.29)	12.43 ± 3.18 (4.70 - 18.20)	6 (1 - 7)	0.29 ± 0.18 (0.06 - 0.77)	0.45 ± 0.19 (0.07 - 0.81)	2	1
	Autumn Oct, Nov	60 (19 / 41)	13.98 ± 0.73 (12.50 - 15.70)	1.00 ± 0.08 (0.88 - 1.27)	10.83 ± 3.67 (4.40 - 17.20)	4 (1 - 7)	2.09 ± 1.34 (0.16 - 6.66)	0.45 ± 0.19 (0.14 - 0.91)	1	1
Northern Adriatic Sea (GSA 17) Med	Winter Feb	30 (12 / 18)	13.25 ± 0.81 (11.90 - 15.90)	0.96 ± 0.07 (0.83 - 1.13)	6.05 ± 2.48 (3.10 - 12.95)	3 (1 - 6)	3.73 ± 1.89 (1.25 - 8.10)	0.92 ± 0.44 (0.36 - 1.97)	2	2
	Spring May	30 (12 / 18)	12.87 ± 0.46 (12.10 - 13.90)	0.94 ± 0.07 (0.83 - 1.09)	9.52 ± 3.38 (4.30 - 17.40)	3 (1 - 6)	0.37 ± 0.23 (0.12 - 1.03)	0.56 ± 0.26 (0.12 - 1.13)	3	3
	Summer Sep	31 (19 / 12)	13.58 ± 0.78 (12.30 - 15.60)	1.05 ± 0.07 (0.96 - 1.33)	15.78 ± 2.34 (11.05 - 21.35)	7 (6 - 7)	0.39 ± 0.24 (0.06 - 0.90)	0.68 ± 0.31 (0.10 - 1.29)	0	0
	Autumn Nov, Dec	40 (24 / 16)	13.37 ± 0.91 (11.70 - 15.60)	1.06 ± 0.18 (0.85 - 1.56)	8.08 ± 4.37 (3.45 - 16.85)	2 (1 - 6)	3.36 ± 1.58 (0.63 - 6.54)	0.30 ± 0.14 (0.04 - 0.62)	13	12
Aegean Sea (GSA 22) Med	Winter Feb	35 (8 / 27)	12.68 ± 0.70 (11.60 - 15.00)	0.97 ± 0.07 (0.83 - 1.19)	4.75 ± 1.09 (3.30 - 8.25)	1 (1 - 2)	5.79 ± 1.33 (3.19 - 8.73)	0.36 ± 0.28 (0.06 - 1.18)	0	0
	Spring	31	12.78 ± 0.56		9.36 ± 2.98	5			0	0

Jun	27	4	(11.90 - 14.50)	0.99 ± 0.06 (0.85 - 1.16)	(4.35 - 16.05)	(2 - 6)	0.41 ± 0.35 (0.10 - 2.18)	0.71 ± 0.26 (0.31 - 1.28)		
Summer Jul	30	19 11	13.63 ± 0.57 (12.30 - 15.00)	1.07 ± 0.06 (0.94 - 1.19)	17.35 ± 3.29 (10.85 - 24.35)	6 (2 - 7)	0.33 ± 0.16 (0.10 - 0.62)	0.78 ± 0.24 (0.30 - 1.34)	0	0
Autumn Oct	35	23 11	12.52 ± 1.21 (10.20 - 14.90)	0.98 ± 0.06 (0.86 - 1.16)	7.94 ± 3.35 (2.05 - 15.95)	3 (1 - 7)	0.45 ± 0.26 (0.09 - 1.10)	0.28 ± 0.14 (0.09 - 0.73)	0	0

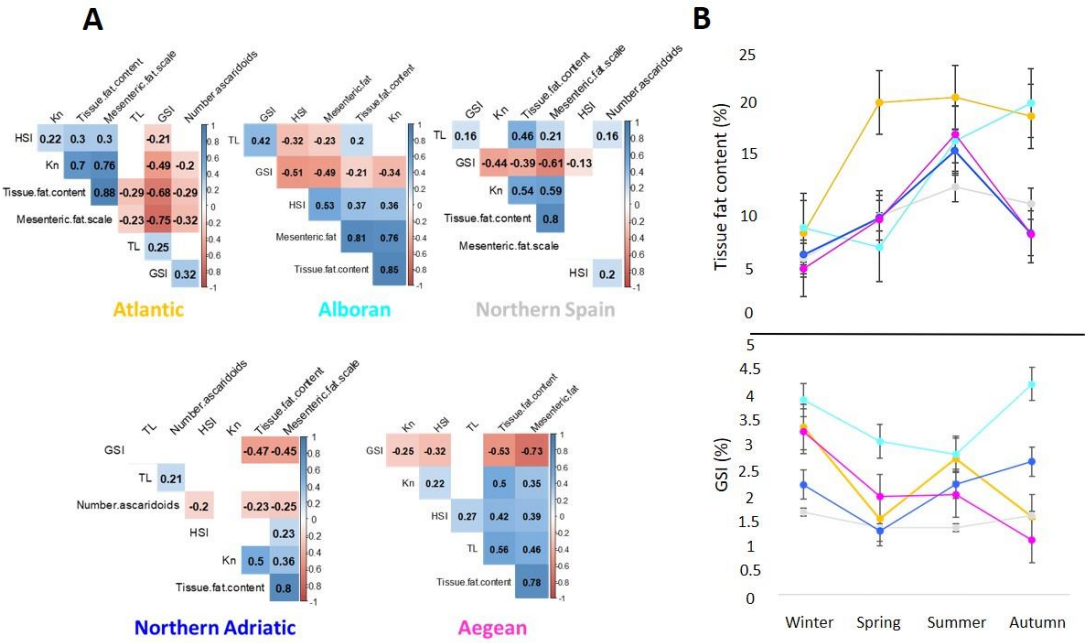

Figure 3. A. General picture of the Spearman correlation matrixes among the European sardine's (*Sardina pilchardus*) condition indices, including the abundance of ascaridoid nematodes in the parasitised stocks (Atlantic, Northern Spain and Northern Adriatic). Total length (L_T; cm), relative condition index (Kn), tissue fat content (%), mesenteric fat scale, gonadosomatic index (GSI; %), and hepatosomatic index (HSI; %) were related among each other and with the number of parasites per fish. The colour gradient from maroon to dark blue corresponds to the correlation with strength, from negative to positive, respectively. The empty squares represent a non-significant correlation according to a p-value of < 0.05*. **B.** Annual trends of tissue fat content (%) and GSI (%). Orange: FAO Division 27.9. a Portuguese Waters - East; blue: GSA 1 Alboran; light grey: GSA 6 Northern Spain; electric blue: GSA 17 Northern Adriatic; pink: GSA 22 Aegean.

We found the largest range of actively spawners in winter (Figure 4A), although individuals in active spawning were detected in autumn for the Alboran (13.3 %), Northern Spain (48.3 %) and the Northern Adriatic (85 %). Moreover, in Alboran it was detected a high percentage of active spawners in spring (71.4 %), and some individuals at this stage in the Aegean (3.2 %), both non-infected locations. In the Atlantic and the Northern Spain, the ratio of infection by sex was similar in both sexes (χ^2 = 0.6975, *df* = 1, p-value = 0.4036; and χ^2 = 0.1652, *df* = 1, p-value = 0.6844, respectively). However, in the Northern Adriatic, more males than females were infected (χ^2 = 7.3351, *df* = 1, p-value = 0.0067*). Among the infected sardines in the Atlantic, the 100 % of them were active spawners (Figure 4B). In Northern Spain, we found that the 50 % of the parasitised individuals were at regressing gonadal phase, 40 % at regenerating and a 10 % were spawning capable individuals, but no significant difference was proven (χ^2 = 2.6, *df* = 2, p-value = 0.2725). In the Northern Adriatic, 70 % were at actively spawning phase, 10 % were in gonadal regression and 20 % at regenerating stage, existing significant differences among the probability of belonging to one of these stages when parasitised (χ^2 = 6.2, *df* = 2, p-value = 0.0451*).

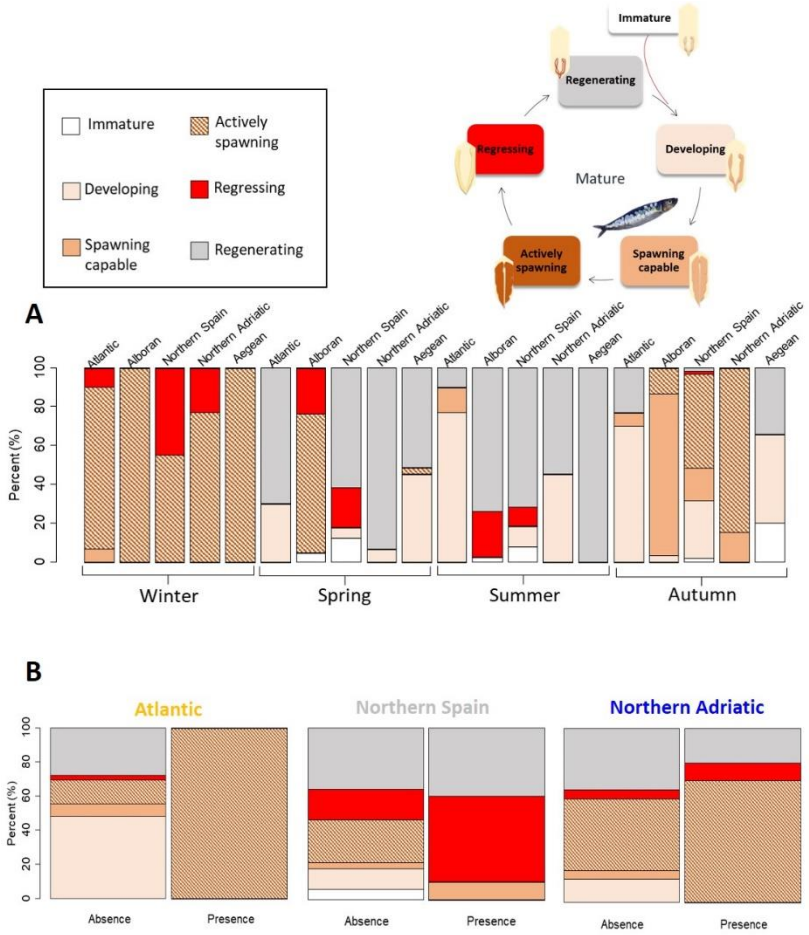

Figure 4. A. Seasonal variations in the frequency of the reproductive developmental stages (those defined by Brown-Peterson et al., 2011) of European sardine (*Sardina pilchardus*) in the stocks analysed. **B.** Presence and absence of parasitism by reproductive developmental stage in the stocks with ascaridoids prevalence. Atlantic: FAO Division 27.9.a Portuguese

Waters - East; Alboran: GSA 1; Northern Spain: GSA 6; Northern Adriatic: GSA 17; Aegean: GSA 22.

Differences in length have been found among sardines from different fishing grounds (Figure 5) (min. total length of 9.5 cm in Northern Spain and max. total length of 20.5 cm in the Alboran). Mean values of total length were the highest in sardines from the Atlantic and the Alboran, while sardines of the Aegean presented the lowest values. Intermediate sizes were detected in the Adriatic and the Northern Spain ($F = 524.69$, p-value < 2.2e-16*). The minimum total length at which parasites were detected was 12.6 cm in individuals from the Adriatic. Bigger sardines in the Northern Spain coincided with the parasitised, while in the Atlantic and the Northern Adriatic this was not observed, although infected sardines were larger in both cases. However, the number of nematodes was positively linked to the total length in the Adriatic and the Northern Spain (see S5.1 for statistics).

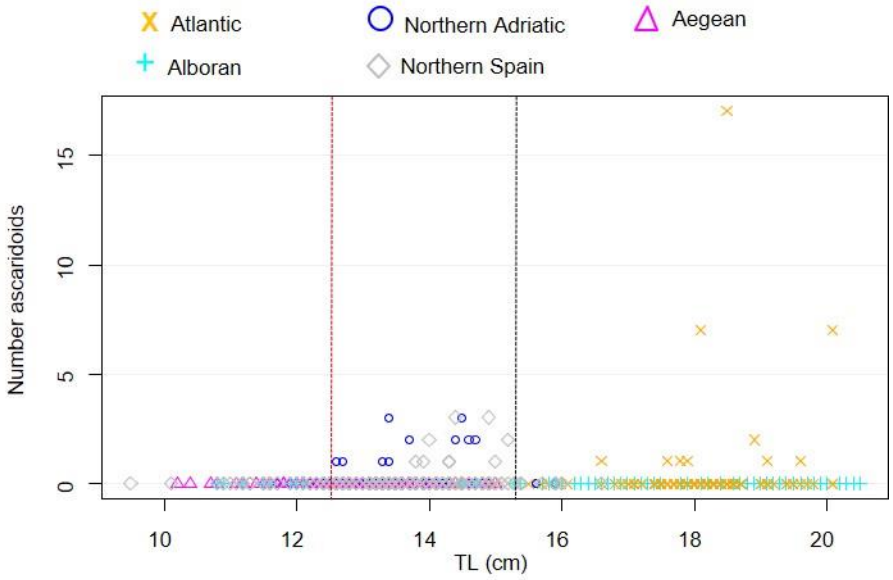

Figure 5. Plot of European sardine (*Sardina pilchardus*) individuals by total length (cm) and its correlation with the number of ascaridoids. Orange: FAO Division 27.9._a Portuguese Waters - East; blue: GSA 1 Alboran; light grey: GSA 6 Northern Spain; electric blue: GSA 17 Northern Adriatic; pink: GSA 22 Aegean. The red dotted line shows the lowest sardine size parasitised; the black dotted line indicates the critical length from which the differences in the number of parasites cease to be significant.

Exploring the relationship between parasitism and total length seasonally in each locality, no significant differences were observed in the Atlantic in winter between parasitised and non-parasitised, with no correlation between abundance and sardine size (see Table S5.2) (Figure 3A). Significant differences in size between infected and uninfected sardines were recorded in the Northern Spain in spring, with bigger sardines infected

(mean of 14.51 cm for infected and 13.16 cm for uninfected sardines), and also presenting a positive correlation between size and the number of parasites in this season. In the Northern Adriatic, larger sardines in autumn presented parasites (mean of 14.17 cm) when compared to uninfected individuals (mean of 13.22 cm). Moreover, in this season in the Adriatic there was a positive correlation between the number of parasites and the sardine's length.

The presence/absence and the number of nematodes seemed to be negatively related with the tissue fat content in the Atlantic, and the Northern Adriatic. However, no significant differences were shown in tissue fat content between individuals parasitised and non-parasitised in winter for the Atlantic, for Northern Spain in spring, or for Northern Adriatic in autumn, which was also confirmed by the correlation analysis at seasonal level in parasitised sardines of the Atlantic in winter, the Adriatic in autumn, and the Northern Spain in spring. Similarly, the correlation of the number of nematodes with the mesenteric fat (strongly related with the tissue fat content, see Figure 3) showed a strong negative relationship in the Atlantic and in the Northern Adriatic, but it was not present in the Northern Spain. Nevertheless, no signification was seen when comparing mesenteric fat stage in parasitised and non-parasitised sardines in winter for the Atlantic, similar to what occurred in spring in the Northern Spain, or in autumn in the Adriatic.

Significant differences in relative condition index (Kn) were observed in the Atlantic between parasitised and non-parasitised sardines, reinforced by the Spearman correlation test, which indicated that there was a significant negative relationship between the number of nematodes and Kn for the Atlantic. Nevertheless, in winter in the Atlantic there were not significant different Kn means in infected and non-infected specimens. General values showed that no differences in Kn existed between parasitised and non-parasitised individuals in the Northern Spain. Also, in spring in the Northern Spain, Kn did not significantly vary when parasitised. In the Northern Adriatic, no differences in Kn appeared when comparing infected and non-infected sardines at global level. Nevertheless, significantly lower Kn values were seen in autumn in infected sardines of the Northern Adriatic, with values under the threshold of the good condition (0.97 (< 1)), when non-parasitised reached this value (mean of 1.08). However, when exploring the correlation between the number of ascaridoids and Kn in autumn in the Northern Adriatic, it was not significant.

The number of nematodes was not related to the HSI in the Atlantic, but it seemed to be positively related in Northern Spain, and negatively in the Adriatic. Furthermore, no significant differences were found in HSI between individuals parasitised and non-

parasitised in winter for the Atlantic. Also, significantly different means were quantified neither in autumn for Adriatic nor in spring for the Northern Spain.

Spearman correlation indicated a strong significant positive relationship between the number of nematodes and GSI in the Atlantic, but it was not observed for the Northern Spain or the Adriatic. Significantly higher GSI values were recorded in parasitised individuals in winter in the Atlantic (averages of 7.71 % in parasitised and 5.11 % in non-parasitised), accompanied by a positive correlation when studied in this concrete season. Higher GSI figures were recorded in spring in infected sardine from the Northern Spain, but without significance (mean of 0.40 % in parasitised individuals and 0.32 % in non-parasitised), and no correlation between GSI and the number of parasites established. A slight difference in GSI between individuals parasitised and non-parasitised was observed in autumn for Adriatic, although no significant (averages of 3.76 % and 3.29 %, respectively, and no correlation between GSI and ascaridoid abundance.

5.4. Discussion

In this study we characterised the ascaridoid parasites of the commercial small pelagic fish *Sardina pilchardus* in different fishing stocks along the Mediterranean Sea and one from the Atlantic Ocean, the south-western Iberian Peninsula. To obtain a complete picture of the parasitisation throughout each whole individual, UV-press was used to detect the ascaridoids. This method has been considered, together with peptic digestion, the only procedures capable of providing a standardised estimate of larval ascaridoid localisation in the fish host as, in fact, the efficiency of candling and visual inspection in the detection of larvae tends to be low (Mattiucci et al., 2018). By applying this technique, our results revealed that only the third larval stage (L3) of ascaridoids was found, appearing in the digestive system and, mostly, in the pyloric caeca in all the stocks and seasons explored. We could confirm that no larva (neither *H. aduncum* nor *Anisakis* spp.) migrated to the muscle in the sardine individuals sampled. Our results are consistent with Køie (1993) and Balbuena et al. (2000), since these investigations pointed that L3 of *H. aduncum* is the larval stage mainly found in the mesenteries and digestive tract of intermediate fish hosts, like sardines, which require at least another intermediate host, a crustacean, for its transmission to fish. The fourth larva (L4) and adult stages of *Hysterothylacium* accumulate in the stomach and intestine lumens of definitive hosts (Deardorff & Overstreet, 1981; Køie, 1993; Navone et al., 1998). In the fish stomach, larvae are activated and penetrate the stomach wall in order to seek residence in the peritoneal cavity, musculature or organs such as liver (Buchmann & Mehrdana, 2016). However, it has been also suggested that the propensity of ascaridoid larvae to migrate to the flesh may be related to differences in the nature (such as

fatty acid content) of the flesh across fish species (Mattiucci et al., 2018). In fact, Smith (1984) reported that larvae migrate post mortem into the flesh of 'fatty' species, as sardine, but not of 'non-fatty' species. Thus, not having found ascarioids in sardines' digestive system could be explained because fish samples were immediately frozen after capture, having existed a short time for the migration to have occurred. Nevertheless, we cannot ensure that the sardine individuals would not have manifested these parasites in flesh a posteriori, with the problems that this would entail at the level of sale and consumption, especially of the fish from the stocks in which Anisakis spp. were found (South Portugal FAO Division 27.9.a and Northern Adriatic). This is because the main ascaridoid identified, *H. aduncum*, has been described as a species with neglectable/little allergenic risk for humans (Cavallero et al., 2015, 2020), although some analysis demonstrated that it shares allergens in common with the genus Anisakis, being associated with non-invasive anisakidosis and hypersensitive responses (Malagón et al., 2010).

For this reason, accurate identification at the species level is important to understand epidemiological patterns, but also biological and ecological, so that morphological methods are useful but often insufficient for specific identification (Roca-Geronès et al., 2018a). Thus, to assign the larvae ascaridoids to species, it was necessary to make use of molecular identification (Mattiucci & Nascetti, 2008; Aibinu et al., 2019; Pekmezci & Yardimci, 2019; Roca-Geronès et al., 2020). In fact, it was reported that visual detection at species level in *Hysterothylacium* spp. is difficult to be carried out as larval types are morphologically indistinguishable (Roca-Geronès et al., 2018b). Moreover, the inconsistency in morphological features of Anisakis species prompted the need to classify these nematodes by genetic and/or biochemical methods (Mattiucci & Nascetti, 2006).

Anisakis spp. and *Hysterothylacium* spp. have been previously reported in sardines from the Atlantic and Mediterranean basin (Rello et al., 2008; Gutiérrez-Galindo et al., 2010; Mladineo & Poljak, 2014; Serracca et al., 2014; Cavallero et al., 2015; Molina-Fernández et al., 2015; Bušelić et al., 2018; Frigola-Tepe et al., 2022). The comparison of these results reveals variable prevalence in sardines originating from different fishing areas, as confirmed by our study, in which, despite the differences among stocks, parasitic indices were generally low for both genus throughout the analysed area. The ecology and behaviour of the host, but also the interplay of host-parasite and host-ecosystem interactions (Brunner et al., 2017) are the responsible for the parasitic variability in fish by species, population, and region, as well as for the divergence of parasitisation in the same fish stock throughout the year. Thus, previous studies have highlighted that the prevalence of parasites in whole fish and fillets is influenced by the season, observed for *H. aduncum* and *A. simplex* (Strømnes & Andersen, 2000; Gay et al., 2018).

Plankton composition, the behaviour and distribution of the nematodes in the variable oceanographic context (i.e., the presence of upwelling or downwelling conditions) (Mattiucci et al., 2018), as well as the feeding habits of the zooplankton consumers throughout its annual cycle may condition the parasitic load in fish. Therefore, the fact that the most frequent ascaridoid in European sardine as intermediate host is *H. aduncum* and that the fish species is infrequently infected by Anisakis along the studied distribution suggests that, on the one hand, the invertebrate hosts of the two parasites are different and/or, on the other hand, the zooplankton from the upper layers are less parasitised by Anisakis, resulting in less parasitisation of pelagic clupeids (Rello et al., 2008).

In this line, no *Anisakis* spp. were characterised in sardines from the Northern Spanish Coast (GSA 6), supported by previous studies in the area (Rello et al., 2008; Gutiérrez-Galindo et al., 2010; Frigola-Tepe et al., 2022), and contradicting analyses based on morphological identification (Biton-Porsmoguer et al., 2020). Nevertheless, *H. aduncum* was observed throughout the whole year, except in winter in this location, and with much lower total prevalence and mean intensity than the 25.21 % and the 2.10 obtained by Rello et al. (2008). A higher prevalence in spring was detected (9.3 %), coinciding with the late winter-early spring planktonic blooms in the north-western Mediterranean (Vidussi et al., 2000; Saiz et al., 2014), to which a principal peak of zooplankton mainly constituted by copepods (72 %–94 % of the total zooplankton) is associated (Puelles et al., 2003). Moreover, water column seasonal stratification typically starts around April (Saiz et al., 2014), which has been shown to facilitate the transfer of *H. aduncum*, as the availability of suitable intermediate and final hosts is highest under these conditions (Klimpel & Rückert, 2005). Likewise, after the spawning period of sardine involving autumn and winter in GSA 6 (Figure 4A), individuals started to feed intensively to recover from the reproduction investment, as well as to start to accumulate reserves for the next laying season, following the capital breeder strategy (McBride et al., 2015). In this way, it could be expected that the ability to acquire parasites through diet at the beginning of the reserve storage season is higher, as reflected in our results.

Both *A. pegreffii* and *H. aduncum* were also detected in sardine by Cavallero et al. (2015) in the Northern Adriatic Sea (2011–2012), with higher prevalence of *H. aduncum* than the reported in our analysis (42.4 % and 7.6 %, respectively) but lower prevalence values of *A. pegreffii* (0.2 % and 0.8 %, respectively). However, Bušelić et al. (2018) recorded a prevalence of 6.0 % in 2013 of *A. pegreffii*, with a significant decrease in 2014 (1.0 %), similar to the value obtained. Comparing to the Northern Spanish Coast, ascaridoid prevalence in the Northern Adriatic was significantly higher, probably related to production, as the Northern Adriatic Sea system is one of the major chlorophyll hot spots in

the Mediterranean Sea, and it has been recognised to depend on the water and nutrient discharge from the Po River and a dozen small rivers that flow into the Adriatic Sea with a major impact on phytoplankton biomass due to the nutrients loads (Caballero-Huertas et al., 2022b).

Moreover, no individual of the genus *Anisakis* or *Hysterothylacium* were observed in the Aegean Sea throughout the year, diverging from the visual results obtained by Kuran et al. (2021), in which they observed L3 of A. simplex and adult individuals of *H. aduncum* in sardines. Chaligiannis et al. (2012) molecularly identified the presence of A. pegreffii with low prevalence (5.5 %), and without signal of infection by the rest of ascaridoids analysed in this study. One potential explanation to this event may be the differences in sardine's trophic ecology among localities, as it has been documented that in the Northern Aegean Sea, sardine's diet is numerically dominated by phytoplankton (Nikolioudakis et al., 2012), while in the Atlantic and other Mediterranean locations most of their dietary energy derives from zooplankton with an occasionally substantial consumption of phytoplankton (Chen et al., 2021). In this way, the lower presence in its diet of the host prior to sardine (copepods and other crustaceans) in the area could be conditioning the intake of ascaridoids.

Ascaridoid absence in sardine occurred as well in the Alboran, as no nematode was visualised in this location. In agreement with the results of Molina-Fernández et al. (2015), neither *Anisakis* nor *Hysterothylacium* larvae were found in fish from the Alboran, which also coincided with the rate of infection in anchovy in this GSA for *A. pegreffii* (Cipriani et al., 2018). This may be supported by the fact that Alboran region is not consider a main area of downwelling (Waldman et al., 2018), unlike other locations in the Mediterranean Sea, which is known that this oceanographic event provides optimal conditions for successful recruitment of the nematode larval stage to pass to the successive intermediate/paratenic hosts (Gregori et al., 2015).

Despite its closeness with the Alboran, mainly *H. aduncum* but also species of *Anisakis*, *A. pegreffii* and *A. simplex* (s.s.) were identified in the Atlantic waters of the Southern Iberia. Rello et al. (2008) found a prevalence, intensity, and abundance of 3.40 %, 1.60 and 0.05 for *H. aduncum*, lower than the reflected by our results (7.5 %, 3.889 and 0.292, respectively). This apparent growth in the infection rate could be due to temporary changes in the climate and its consequent effect on the parasite community and/or the host feeding behaviour. Nevertheless, it cannot be confirmed since the sampling season for the mentioned study is unknown and this could be conditioning the comparison between results. By contrast, the authors recognised this area as sympatric for *A. pegreffii* and *A. simplex* (s.s.) after finding these nematodes in anchovy, although the authors did not observe any of these species in

sardine. This sympatric event, as well as some hybridization between the anisakids, have been previously described (Mattiucci et al., 2016; Bello et al., 2021; Roca-Geronès et al., 2021). In this stock, the highest ascaridoid intensity and abundance were characterised. As it could be observed (Figure 5), it consisted in the parasitised stock with bigger sardines, and with the highest accumulation of fat throughout the year (Figure 3B), sign of an abundance of resources in the area (Caballero-Huertas et al., 2022c) and a different dietary strategy (Costalago et al., 2015). Assuming that fish size increases with resources availability and age, larger and older fish are expected to accumulate a larger number of larval forms of parasites (Aco Alburqueque et al., 2020; Santoro et al., 2021). Nevertheless, total length did not seem to explain the differences in parasitism at intra-stock level in the big sardines of the Atlantic. Thus, we hypothesised that there is a critical size (>15 cm total length) in which length does not determine the amount of ascaridoids present in the sardine individual for the parasitised stocks. At intra-stock level by season, both Northern Adriatic and Northern Spain presented significant larger sizes when parasitised, with a significant positive correlation between the number of nematodes and the total length, which could reflect that in the case of stocks with medium sizes, small variations in size could be decisive in relation to their parasitic load. Thus, the low prevalence of the parasite in small, young sardines may be due to fish larvae and juvenile phenotypes feed preferentially on phytoplankton and, consequently, the youngest fish sent to market have had less opportunity than the longer ones to become infected (Rello et al., 2008).

A notable number of studies have used fish condition indices to monitor and investigate pelagic fish population health and variability (e.g., Brosset et al., 2015b; Albo-Puigserver et al., 2020; Caballero-Huertas et al., 2022c). However, there are not many works that analyse nematode parasitism in the context of condition in these species, and that include the stock variability in the parasitological study of a specific species. In this context, our work has attempted to shed light on the condition of the stocks and their relationship with parasitism, as well as to study whether the individual condition of the individuals in each area can be linked to the presence of ascaridoids. In the clupeoid anchovy, lower lipid content is related with a higher intensity of certain parasites as digeneans and nematodes (Ferrer-Maza et al., 2016), also found by Frigola-Tepe et al. (2022) in sardine. Moreover, the body condition of infected cod was lower than that of those free of parasites and declined with the intensity of infection; the condition of most infected fish was up to 20 % lower than that of uninfected individuals (Horbowy et al., 2016). *A. simplex* infections may be associated with the loss of condition of fish hosts, but in cases where the larvae are sequestered outside essential organs the effect may be less harmful (Buchmann & Mehrdana, 2016). Nevertheless, in our study, an apparent negative relationship could be found in terms of

energy condition parameters in the parasitised stocks (Kn, and tissue and mesenteric fat in the Atlantic, and tissue and mesenteric fat in Northern Adriatic). However, when exploring at seasonal level, no differences between infected and non-infected individuals were observed in condition parameters (except for Kn for the Northern Adriatic), as well as no relationship between the abundance of parasites and fish energetic status was obtained. This reflects that the apparent relationship would be linked to seasonality and the time that the individuals of each stock go through. Figure 4B shows clearly the link between the reproductive stage of the sardine and parasitisation, observing that sardines in active spawning and post-spawning (regressing and regenerating) were the ones that had ascaridoids of the parasitised stocks. It is noteworthy that the two non-parasitised stocks (Alboran and Aegean) corresponded to the sardines that continue to be reproductively active in spring (Figure 3B (GSI) and 4A), so we speculate that in addition to the possible environmental explanations in the areas, perhaps this has to do with the eating habits of individuals, which could be feeding in a different way in terms of intensity and organisms by having to allocate those resources to the later egg laying. If the period of intensive feeding were closer to autumn-winter (instead of spring-summer), the main zooplanktonic bloom would not have taken place yet, in addition to the fact that water stratification and temperature would be lower, conditions that do not favour the cycle of ascaridoids or their transmission between hosts. In fact, previous studies documented the peaks of infection of *H. aduncum* and *A. simplex* in various fish species throughout the spring (Andersen, 1993; Strømnes & Andersen, 2000), moment in which actively reproducing sardines would not be feeding in the same way as the stocks that have finished spawning and are in the post-spawning recovery period (Atlantic, Northern Spain, and Adriatic).

In this work we have seen that there are multiple factors that can affect parasitisation by ascaridoids, mainly related to the ecology of the host species, its annual cycle and its way of accumulating reserves which will be destined to reproduction, which is very notable in capital breeders due to its marked seasonality. It is for all these reasons that the variability in the condition of individuals and parasitism are related due to the environmental characteristics and life history of the stocks, which determine their growth, their mode of feeding and reproducing and, consequently, the load and species of parasites that colonise them as intermediate hosts.

Chapter 6

Evidence of trilobed testes in European sardine (*Sardina pilchardus*)

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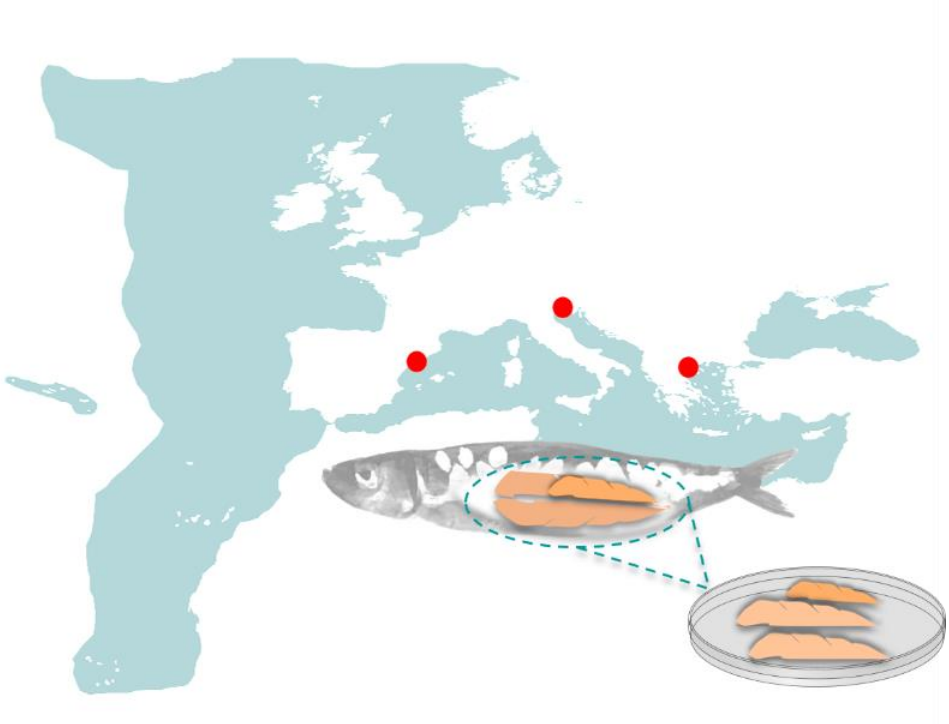

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Abstract

Abnormal gonads were observed in male specimens of European sardine (*Sardina pilchardus* (Walbaum, 1792)) captured along the Mediterranean Coast (Northern Spain, Northern Adriatic Sea, and Aegean Sea). Three lobes were identified in the testes of these individuals, what could be considered as gonad segmentation. The prevalence of this abnormality of the testicles' architecture has reached 2 %, 0.99 %, and 0.49 %, respectively, in the samples of the stocks mentioned above. To knowledge, it is the first documentation of such testicular alteration in this species. Chemical exposure or parasitism have been invoked as plausible hypotheses, although further studies are required to elucidate its cause in this valuable fish resource, since reproductive abnormalities may result in severe implications, including population losses.

Keywords: Abnormal gonads • Gonad segmentation • Pelagic fish • Pilchard • Reproduction • Reproductive pathology

6.1. Introduction

Morphological abnormalities of the gonads have been reported from some wild fish populations. Notwithstanding, because of the difficulties in determining the causative factors and processes, there is a significant lack of knowledge regarding these reproductive irregularities, especially in the marine environment. The term of "abnormal testes" after macroscopical evaluation in bony fishes has been defined as reproductive tissue of testes partly turned into adipose or its development in only one lobe (Domínguez-Petit et al., 2017). However, other types of testicular malformations affecting the number of lobes have been described. Gonad segmentation, termed as "discontinuos" or "multiple" gonads, has been defined as an abnormal fragmentation of the gonadal tissue with an appearance of multiple gonads or segmented gonads into subunits divided by connective or non-gonadal tissue (Hecker et al., 2006). The objective of this study was to characterize and report the testicular abnormality found in individuals of European sardine from three locations along the Mediterranean Sea to provide baseline information for their further monitoring and promote studies focused on its aetiology.

6.2. Materials and methods

Specimens of European sardine (*Sardina pilchardus*) obtained from fish markets were caught between 2019 and 2020 in three Geographical Subareas (GSAs) of the Mediterranean (Figure 1): Northern Spain (GSA 6) n (number of individuals with the testicular abnormality) = 2 (N = 100), Northern Adriatic Sea (GSA 17) n = 2 (N = 203), and Aegean Sea (GSA 22) n = 1 (N = 206).

Morphogravimetric parameters were measured: total length (L_T ± 0.1 cm), total weight (W_T ± 0.01 g), eviscerated weight (W_E ± 0.01 g), and gonad weight (W_G ± 0.0001 g). Gonadosomatic index (GSI) (%) was calculated as an estimation of reproductive investment, using the gonad weight (W_G) (g) and eviscerated weight (W_E) (g) as follows: GSI = 100 x [W_G / W_E]. The relative condition index (Le Cren, Kn), as index of physiological or energetic condition, was computed with the equation and parameters (α and β) applied by Brosset et al. (2015) for European sardine (Kn = W_E / $\alpha L_T \beta$), where W_E is eviscerated weight, L_T is total length, and α and β are constants obtained by the regression line of the logarithms of length and mass.

The sex of each individual was determined macroscopically and gonads were classified according to their reproductive developmental phase following Brown-Peterson et al. (2011). Afterwards, the functionality of the testicles was confirmed with histology. Gonads were fixed in 4% formaldehyde buffered with $Na_2HPO4*2H_2O$ (0.046 M) and $NaH_2PO_4*H_20$ (0.029 M), and a subsample from the central section of all lobes was extracted, dehydrated and embedded in paraffin to be sectioned at 3 µm and stained with haematoxylin–eosin for its examination under microscope.

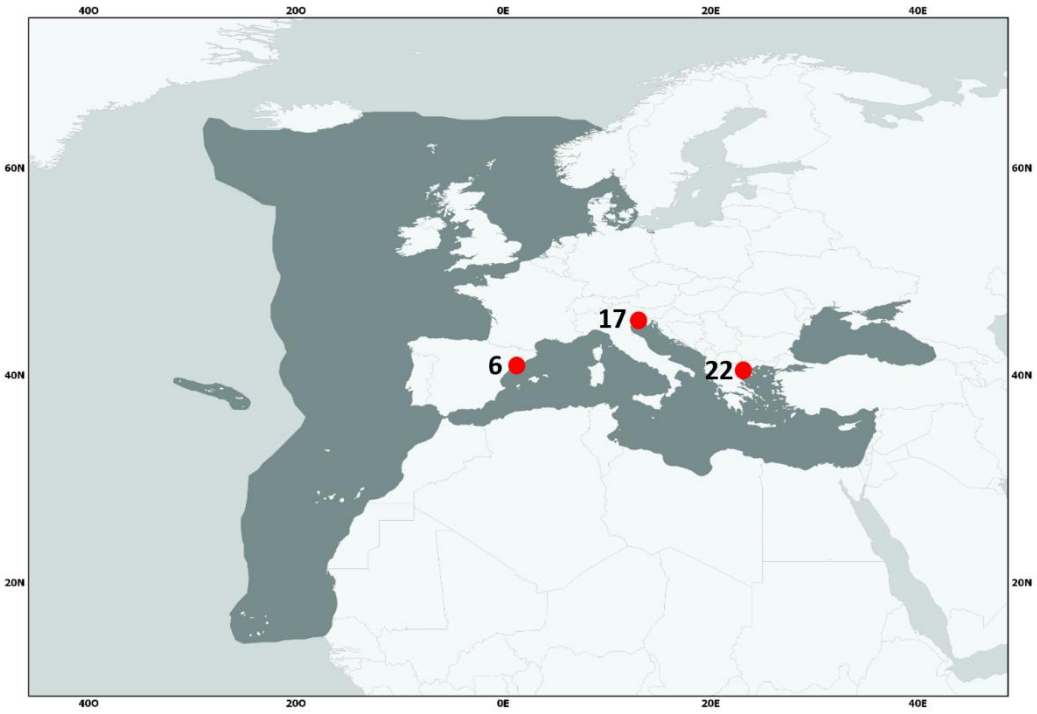


Figure 1. Map of the sampling locations. Numbers refer to the Geographical Subareas (GSAs) of the Mediterranean in which samples were collected. The darker shade corresponds to the distributional range of the European sardine (*Sardina pilchardus*).

6.3. Results

Alterations in the normal gonad anatomy and architecture of male individuals of European sardine, *Sardina pilchardus*, were registered in five of 509 specimens analysed (total prevalence of 0.98%): Northern Spain (GSA 6) n = 2 (N = 100, local prevalence of 2 %), Northern Adriatic Sea (GSA 17) n = 2 (N = 203, local prevalence of 0.99 %), and Aegean Sea (GSA 22) n = 1 (N = 206, local prevalence of 0.49 %) (GSA average prevalence ± SD of 1.16 ± 0.63 %) (Table 1). Gonadal gross morphology analysis revealed that these five individuals presented three asymmetric testicular lobes (Figure 2A, B, C). Individuals had two main gonad lobes and an additional one of smaller size, the latter adopting a rope-like

shape overlapping one of the two main lobes, and weakly connected to the posterior part, which constitutes the spermatic duct. Two males (Northern Spain 15.1 cm L_T (Figure 2B) and Aegean 15 cm L_T (Figure 2A)) showed to be at developing reproductive phase, characterised by easily identified testes close to reach the spawning capability, while the remaining three individuals (Northern Spain 13.9 cm L_T, and Northern Adriatic 13.7 and 13.8 cm L_T) were at spawning capable phase (actively spawning subphase), with firm testes where accumulation of sperm in the spermatic ducts was macroscopically visible (e.g. Figure 2C, D, E, F). In the latter, the sperm was macroscopically detected in a disposition and quantity similar to that of normal testicles in all lobes, supported by the histological analysis, that revealed the presence of spermatids and the release of sperm by all the subunits, including the additional of smaller size.

Table 1. Description of the five individuals of European pilchard (*Sardina pilchardus*) with three-lobed testes analysed. Location (Geographical Subarea (GSA)) and date of capture of the specimens, condition indexes (GSI and Kn), and brief description of morphometric and reproductive traits. Total length (L_T ± 0.1 cm); total weight (W_T ± 0.01 g); eviscerated weight (W_E ± 0.01 g); gonad weight (W_G ± 0.0001 g); gonadosomatic index (GSI); Le Cren condition factor (Kn).

GSA	Capture date	Sex	Reproductive phase	L_T (cm)	W_T (g)	W_E (g)	W_G (g)	Condition indexes	
								GSI	Kn
Northern Spain (6)	5 Feb 2020	Male	Developing	15.1	22.7	20.7	0.4942	2.39	0.92
Northern Spain (6)	6 Mar 2020	Male	Actively spawning	13.9	19.4	17.5	0.3669	2.10	1.04
Northern Adriatic Sea (17)	14 Feb 2020	Male	Actively spawning	13.7	18.2	16	0.4917	3.07	1.02
Northern Adriatic Sea (17)	19 Dec 2019	Male	Actively spawning	13.8	17.8	15.9	0.5000	3.14	0.97
Aegean Sea (22)	16 Nov 2019	Male	Developing	15.0	27.5	24.5	1.3020	5.31	1.14

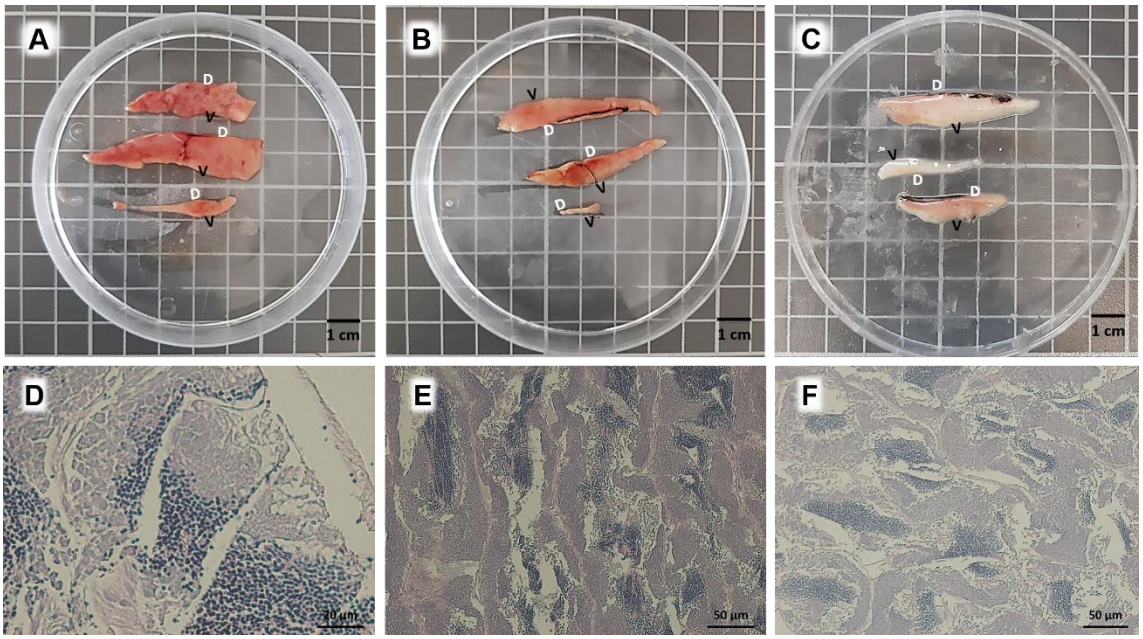


Figure 2. Three-lobed testes of three specimens of *Sardina pilchardus*. **A.** Testicular lobes from a male specimen (15.0 cm L_T) captured in the Aegean Sea (GSA 22); **B.** Testicular lobes from a male specimen (15.1 cm L_T) captured off the Northern Spanish Coast (GSA 6); **C.** Testicular lobes from a male specimen (13.7 cm L_T) captured in the Northern Adriatic Sea (GSA 17). Ventral (V) and dorsal (D) sides of each testicle/subunit are indicated; **D, E, F.** Histological view of the testicular tissue of the individual from the Northern Adriatic Sea (13.7 cm L_T). Figures D and E correspond to the main lobes, and F to the subunit of smaller size.

6.4. Discussion

Irregularities in the development of the reproduction system may occur as early as the stage of gonad formation (Hliwa et al., 2011). Since fish sexual differentiation and gonad development are subject to genetic and environmental influences, alterations in gonad morphology are potentially caused by both endogenous and exogenous factors as well as their interaction by different processes, among which we should highlight the genetic quality and hybridization status of the broodstock, autoimmune disease, chemical exposure (Sangalang et al., 1981; Hliwa et al., 2011; Rajasilta et al., 2016), and parasitic infection (Hliwa et al., 2011) or pathogens (Bernet et al., 2020).

Triplicate gonads appeared in a male individual of sardine-related species Baltic herring (*Clupea harengus membras*) in 2013, and although not robust explanation has been found, the authors suggested that it could be linked with the chronic exposure to environmental contaminants disturbing the endocrine system (Rajasilta et al., 2016). Pollutant agent Aroclor 1254, a polychlorinated biphenyl (PCB), revealed various testicular abnormalities in 9 of 17 PCB-fed cod *Gadus morhua*, which included disorganization of

lobules and spermatogenic elements, inhibition of spermatogenesis, fibrosis of lobule walls, fatty necrosis, and, in one case, total disintegration of the elements in many lobules (Sangalang et al., 1981). Moreover, gonad segmentation, defined by Hliwa et al. (2011) as 'segregation of lobes into subunits' was observed in fish *Coregonus* spp., as well as adherence or adhesion of lobes to the peritoneal wall and lateral muscles, distinct asymmetry in size of both gonads, atrophy, and even hermaphroditism in individuals of some alpine lakes in Switzerland and of water bodies in northern Poland. However, until the moment it has not been supported a chemical aetiology of the gonad malformations. On the other hand, there is not much bibliography regarding the sexual phenotypic effects of parasitic infections suffered along gonadal differentiation in fish. Notwithstanding, it has to be pointed out that Ichalal et al. (2019) linked Ascaridoidea nematode (specifically, Anisakid) infestation with ovarian abnormalities in Atlantic horse mackerel (*Trachurus trachurus*), involving compartmentation (manifested by the division of the ovary into two lobes) as macroscopic result.

Low GSI has often been linked to pathological alteration of the gonads (Louiz et al., 2009). Our results showed that GSI values for both males (13.7 and 13.8 cm L_T) caught in the Northern Adriatic Sea were lower than the GSI range of 3.6-5.09 of the individuals sampled from December to February at the same GSA (Zorica et al., 2019). However, GSI (5.31) of the individual from the Aegean Sea in November was higher than the estimated by Ganias *et al*. (2007) (lower than 4) in the same GSA during this month. Regarding the specimens from the Spanish Coast, GSI for 15.1 cm L_T male was of 2.39 in February, among the GSI range of values calculated by Brosset et al. (2016) at this time of the year in western Mediterranean (GSA7). Surprisingly, it seemed to be at developing phase, which is not usual at this period of the year in this area, in which individuals are found fully in the spawning season (from November to March). In contrast, specimen of 13.9 cm L_T was an active spawner with a GSI of 2.10, higher than the average values found in March in the western Mediterranean along different sampling periods (Brosset et al., 2016).

Reproductive success is dependent on the reproductive health of the parents through processes that damage gonads (Rajasilta et al., 2016). Thus, reproductive abnormalities may result in severe implications on natural populations, including population losses. Nevertheless, even though it is probable that a low incidence in the populations does not pose a problem for the reproductive capacity of sardine or for its recruitment, its presence must be taken into account and monitored over time and space. Also, since gonad mal-formations and architectural divergence, concretely gonad segmentation, may be linked to contamination or parasite abundance in different locations of the Mediterranean coastal

waters, it is imperative to study in depth this trait in order to define and characterise its potential cause/causes in *S. pilchardus*.

General discussion

Due to the main role in the function and structure of the ecosystem of small pelagic fishes (SPF), as well as the socioeconomic importance of these species, it is of vital importance to have information about the condition and state of health of the stocks. Populations of SPF exhibit extreme fluctuations in abundance and geographic distribution due to the impact of environmental drivers, which are often amplified by anthropogenic influences (Alheit & Peck, 2019). Therefore, the continuous updating of knowledge about these species is very important, especially taking into account the changes in the ecosystems that have been taking place due to direct and indirect human pressures.

One of the species which has experienced a progressive drop in catches and biomass, the condition of individuals and their size in recent decades is the European sardine (*Sardina pilchardus*) in a large part of its distribution, especially in the Mediterranean (Sinovčić et al., 2008; Van Beveren et al., 2014; Brosset et al., 2017; Quattrocchi & Maynou, 2017; Şenbahar et al., 2020; Albo-Puigserver et al., 2021). Despite previous research efforts on this species, many questions were raised regarding the geographic variability in sardine health status and its potential causes, as well as in relation to fluctuations in these parameters over time.

The present thesis attempts to respond to many of them, addressing from sardine genetic variability and natural selection to biotic (parasitism, productivity, etc.) and abiotic (water temperature, wind, etc.) environmental factors that shape the differences among stocks. Likewise, this work evaluates the current state of the species taking into account its reproduction, essential to compare potential populations throughout the year and also to evaluate its reproductive phenology and investment in the production of gametes. The accomplishment of the initial main and specific objectives based on several research questions has resulted in a compilation of interconnected information, which is a first big step towards understanding the problems that this species has been facing, as well as to holistically assess the drivers that intervene in the health of the stocks.

Moreover, the management of the sardine fisheries is a great challenge for the authorities responsible for it, and a serious problem for the people who live directly and indirectly from this resource. In the last decades, the biomass and the state of certain sardine stocks has not been the most suitable, which is worrying in a scenario of thermal increase and derived ecosystem changes. Therefore, the most inclusive perspective from the point of view of the species and the ecosystem, as well as of the sectors involved when making decisions on delimiting fishing areas, establishing fishing quotas and closed seasons, etc. will always be the most appropriate. In this way, I hope that the information and main

conclusions from this thesis contribute to the sustainable management of this important resource.

1. Linking the main findings on the variability of sardine condition and its cause

Biological processes and functions (i.e., growth, maintenance and reproduction) and environmental factors (i.e., temperature, food availability, etc.) shape energy storage and condition of small pelagic fish, determining the health status of the individual itself, of the population and, ultimately, of the ecosystem. Moreover, as condition and reproduction are intertwined (Garrido et al., 2008b; Lloret-Lloret et al., 2022), changes in reproduction are usually related to changes in somatic growth and condition, thus affecting natural mortality and recruitment success, consequence of the trade-off between the energy invested in growth, maintenance, and reproduction (Albo-Puigserver et al., 2021).

To assess the status of a pelagic species and, therefore, carry out proper management, it is important to understand the variability throughout its distribution, emphasizing those areas where the status of the species is most vulnerable. For this purpose, phenotypic plasticity could explain spatio-temporal variability of traits (Menu et al., 2022), although biological units (i.e., genetic variability) and processes (e.g., migration) have to be considered in these studies. However, to define genetic stocks in coastal pelagic marine fishes continues to be a challenge due to several reasons as the apparent lack of physical barriers in the marine environment, the high dispersal capabilities in larval and adult stages (Stern et al., 2018), a large effective population size limiting the impact of genetic drift (Laurent et al., 2007), and the methodological limitations of the molecular markers (Kasapidis, 2014). In the case of the European sardine, the hypotheses of the establishment of the genetic units were diverse, so that we found the lack of a study that encompassed the most probable genetic stocks based on previous research. In this sense, the first chapter (**Chapter 1**) of this thesis proposes for the first time the most plausible sardine stocks, concluding that well-defined populations appear to exist (i.e., North-Atlantic, South-Atlantic, Azores-Madeira and Adriatic-Ionian stocks), with larger than expected diversity estimates in marine fish species. Nevertheless, great uncertainties emerge with regard to the waters surrounding the Iberian Peninsula, from which the westernmost samples were analysed in these study, coinciding with a diverse state of condition, reproductive traits, and even nematode load in sardines that we quantified and discuss in **Chapters 3**, **4** and **5**. In fact, the worst condition status analysed in this thesis was observed in the Catalan Coast

([GSA 6] Northeast Iberian Peninsula, North-western Mediterranean), and the greatest energetic reserves and further condition indices were quantified in the South of Portugal-Gulf of Cádiz (POR-GC) (Southwest Iberian Peninsula), being the stock from the Alboran (Alb) (Southeast Iberian Peninsula) quite similar to the latter although seasonal Kn, tissue and mesenteric fat content, and HSI values of POR-GC specimens exceeded Alboran's with summer arrival, period in which sardine acquires reserves to allocate them to reproduction. The low condition status of the individuals analysed from the Catalan Coast (Northern Spain) has been previously reported in this area (Van Beveren et al., 2014; Albo-Puigserver et al., 2019) and attributed to changes in planktonic dynamics (prey abundance, composition, and quality) as a result of an intense warming in recent decades with which this stock has not been able to deal.

On the other hand, the best state of condition of the individuals from the POR-GC and Alb can be explained by several arguments. Remarkable genetic differences have been documented on one side of the Strait of Gibraltar and on the other, even establishing two different subspecies in the Atlantic and Mediterranean (*S. pilchardus pilchardus* and *S. pilchardus sardina*) (Regan, 1916; Lee, 1961), although this hypothesis does not seem to be supported based on the most recent genetic studies, in which the genetic similarities between the individuals of the Alboran and those of the Atlantic are commented (Coll & Bellido, 2019; Antoniou et al., in press). Previously, bimodal size frequency distributions were described, with larger sizes in the westernmost area of the Alboran, suggesting that the incorporation of sardine individuals of the Atlantic in the north coast of the Alboran was happening (Garcia-Lafuente et al., 2021). However, our study has gone further with the development of a hypothesis based on this aspect supported by condition and reproduction data, in addition to having offered explanations based on ecological and environmental aspects. The colder waters of the Atlantic, which penetrate towards the Alboran, in addition to the high productivity in an area influenced by the nutrients input of important rivers (Arade, Guadiana, and Guadalquivir) and trade winds intensification during summer (months in which European sardine begins to feed intensively) allow a high growth and condition of the fish, leading to a faster maturation of the gonads. The main circulation pattern favours the zonal connectivity East-to-West, which includes the interbasin connection Gulf of Cadiz-Alboran Sea through the Strait (García-Lafuente et al., 2021). In **Chapter 3**, we obtained some evidence that this connectivity between populations could be happening, with the migration of the reproductively mature Atlantic sardines towards the spawning areas in the Alboran, and whose displacement could also be motivated by the advanced drop in local temperatures in this area of the Mediterranean.

In relation to the parasitic load in the Iberian stocks, in **Chapter 5** we observed differences both in the species of nematodes present, as well as in the infection parameters. On the one hand, the only species present in the Catalan Coast (Northern Spain) was *Hysterothylacium aduncum*, with a prevalence of 3.9 and a mean intensity of 1.600 ± 0.843. The stock of the POR-GC, in turn, presented this species with a prevalence and intensity of 7.5 and 3.889 ± 5.278, respectively. Also, two species of the genus *Anisakis*, *A. simplex* (s.s.) and *A. pegreffii*, appeared at low prevalence and intensities at this locality. On the other hand, in the Alboran stock no nematode was quantified throughout the annual cycle of the sardine. These remarkable differences in the parasitic characteristics are related with the ecology and feeding behaviour of the host, as well as with host-parasite co-adaptation and co-evolution, and/or to interspecific competition that can reduce the range of potential hosts in sympatric conditions (Mattiucci & Nascetti, 2008). This use of nematodes helps to the stock delimitation, which is known as biological tagging (MacKenzie et al., 2008). In our study, we conclude that the lack of downwelling in this area (Waldman et al., 2018) does not provide optimal conditions for the success of the nematode larval stage to pass to the successive intermediate/paratenic hosts (Gregori et al., 2015), and that there is a link between the reproductive stage of the sardine and parasite presence, which also diverged between the Alboran (also, the Aegean, another non-parasitised stock) and the rest of the stocks. Alboran and Aegean fish continue to be reproductively active in spring, therefore the period of intensive feeding were closer to autumn-winter instead of spring-summer (common in the rest of stocks), and the main zooplanktonic bloom would not have taken place yet, in addition to the fact that water stratification and temperature would be lower, conditions that hinder the cycle of ascaridoids and/or their transmission between hosts.

In the same way, differences in the prevalence of a testicular anomaly, the appearance of an apparently functional third testicle, was detected in the Catalan Coast (2 %), and not observed in the other two stocks close to the Iberian Peninsula, although it was present in sardines in the North Adriatic (0.99 %) and North Aegean Sea (0.49 %) in **Chapter 6**. In this regard, the origin of this abnormality should continue to be studied (testing the hypotheses linked to genetic causes, autoimmune diseases or chemical exposure), since the hypothesis of infestation by nematodes of the testes is ruled out after UV-press (no parasites detected in gonads and null infection rates in the Aegean), as well as the histology of the gonad, which did not seem to show alterations as a result of the parasitic presence.

Leaving aside the area of the western Mediterranean and its transition with the Atlantic, and focusing on the central Mediterranean stock included in our study, the Northern Adriatic Sea, we have reached certain conclusions that, in addition to helping to describe aspects of the physiology and ecology of this stock, could be applied to the rest of

the species. **Chapter 1** helped us to understand the genetic divergence of the Adriatic-Ionian stock with respect to the rest of Mediterranean individuals, probably due to larval retention and confined dispersal because of the land enclosure caused by the Italian and the Peloponnese Peninsulas (Magoulas et al., 2006). In this sense, data in nematode parasitism (infection levels and species) showed differences compared to the other stocks, which is one more factor that helps to identify this stock as a biological unit or population (Mattiucci & Nascetti, 2008). We observed that it was the most parasitised stock by the species *Hysterothylacium aduncum* (prevalence of 7.6 %, mean intensity 1.700), in addition to being the only Mediterranean stock to present the species *Anisakis pegreffii* (prevalence of 0.8 %). The study by Cipriani et al. (2018) in the clupeoid anchovy (*Engraulis encrasicolus*) also indicates the Adriatic as the area with the highest prevalence of *A. pegreffii*, especially in the south, and practically insignificant in other areas such as Alboran and the Aegean.

The generation of a genetic unit resulting from genetic isolation is also accompanied by its own characteristics in terms of condition and reproduction. In this direction, **Chapter 4** reflected how the condition of this stock was generally above the rest throughout the reproductive period. Moreover, the hepatosomatic index (HSI) also reflected the highest values and a different trend compared to the rest of the stocks (i.e., we observed great livers that started to grow before and during active reproduction). These certainties are probably related with the fact that sardines in this area (Gulf of Trieste) have feeding resources available all year round due to its geographical location within a semi-enclosed basin (Brosset et al., 2017), in addition to the continue deposit of nutrients by rivers, being even more eutrophic at late winter-spring, coinciding with sardine's reproduction. Thereby, we can conclude that the access to continuous resources has an effect on various aspects of the biology of this stock. First, the storage period and the mobilisation of energy destined to the maturation of gametes in the Northern Adriatic is not as marked as it could be in other stocks, so in this case, the current income would be occupying a key role in reproduction. Secondly, big livers are related to an intense feeding activity and, therefore, to fish condition. Thus, although the liver is not the major energy reserve organ in sardines (as tissue and mesenteric fat are), it gives us information about the availability of food and, therefore, about the allocation of energy for processes such as gonadal maturation until the spawning peak, which was the fastest to be reached of all the stocks analysed (also explained by the greatest temperature difference between the winter and summer months, which can also alter the way of storing and allocating energy and trigger the reproductive period). In this regard, in **Chapter 2** we obtained a notable positive correlation between tissue and mesenteric fat content and the available portion of productivity for the fish (OPFish), being lower in the relationship between the HSI and the OPFish, although also significant and

positive. In this sense, feeding resources are related to body condition and fat accumulation with a potential delay. In the central Aegean and Ionian Seas, Ganias et al. (2007) observed a direct correlation between the spring burst of primary productivity in HSI although a lag with respect to somatic condition and mesenteric fat, possibly due to the exportation of energy from the liver to long-term storage sites such as mesenteric adipose tissue and muscle. Therefore, it is possible that the week of capture of the samples within even the same month influences the moment in which the fish are in relation to the destination of their reserves and, therefore, the variations in the correlations between environmental and energy storage parameters. Moreover, HSI and GSI showed practically no correlation (slightly negative in males), sign that the energy contained in the liver and the energy that goes to the gonad is not direct, but rather has to do with the general state of the individual and the energy content in its main reserves (more strongly correlated). Furthermore, there was a clear inverse relationship between the size of the gonads, demonstrated by the GSI, and the temperature, helping to explain the contribution of SST drop in gonad maturation and egg release. Thus, the spawning season duration would result from a synergistic effect of this physical factor and the exhaustion of stored energy (Nunes et al., 2011).

On the other hand, in the Adriatic stock differences were identified between males and females in condition and fat content. Females' status was generally greater than that of the males, and they contained more lipids before spawning until reaching similar values at the beginning of the active reproductive season, also observed in POR-GC. This could be occurring due to a greater energy demand by females when maturating the ovaries, since the requirement of fatty acids from the mother's muscle is related to the development of oocytes (Garrido et al., 2007b). In line with our results, Garrido et al. (2008b) showed that the muscle tissue of male and female sardines during the spawning period did not show significant differences in total fatty acids concentration. However, these clear differences were not observed in other stocks such as Alboran's.

Chapter 3 also contributed to highlighting the importance of studying the condition not only using the Kn index based on length and weight, potentially correlated with protein content or other health indicators (Brosset, 2016), but combining it with fat indices. Campanini et al. (2021) demonstrated the suitability of fatmeter measurements versus Kn index in sardine, what we have verified through the correlation analysis between Kn and both GSI and reproductive stage. Theoretically, the inverse relationship between condition and reproduction is well known (e.g., Ganias et al., 2007; Albo-Puigserver et al., 2020), although Kn does not always numerically reflect this relationship, as has been observed in our results. However, and as explanation of the combination of several indices and variables in this manuscript, it is important to keep in mind the intrinsic limitations in the individual

use of indirect cues or indices, as there may be a time lag between variations in lipid levels and weight, the latter varying more strongly when there are proteins that begin to be consumed and therefore differing when the lipids are already low, giving a delayed signal (Brosset, 2016).

Finally, some aspects regarding the condition of the Northern Aegean stock were the low values from the developing period to the actively spawning. However, it was a great recovery at the regressing phase, being the largest of all the stocks analysed (**Chapter 4**). This event coincided with a peak in primary productivity in the area during the summer, when these individuals were potentially feeding intensely. In fact, sardine modelled biomass peaked during June-July, when optimum somatic condition and increased juvenile abundance coincide (Gkanasos et al., 2021). During the period 1985-2011, the average summer SST in the North Aegean was among the lowest of the Mediterranean (Marbà et al., 2015), despite a large thermal anomaly (Hidalgo et al., 2018). However, we observed the highest value in summer during the years of our study (2019-2021).

Food intake might be limited when high temperatures are above the thermocline in these months (Nikolioudakis et al., 2011). High temperatures also contribute to zooplankton decrease (Gkanasos et al., 2021), which could affect the trophodynamics of the sardine and, therefore, its condition. Moreover, changes in fertility/reproduction and phenology may be triggered by warming (Marbà et al., 2015), by both the direct action and the implication of temperature in the capture, storage and allocation of energy, as we have seen, closely related to the reproductive traits. In fact, a decrease in sardine biomass is expected under the future climate change scenario in the area (Gkanasos et al., 2021), that could be compromising both sardine growth and condition, and recruitment.

2. Applications to fisheries management and assessment

In addition to inquiring into the knowledge of the population structure, state of health and factors involved in the condition and reproduction of European sardine, one of the purposes pursued by this thesis is to provide notions applicable to the management of this important fisheries resource with the intention of improving fishing practices and policies. Including reproductive, body condition, and epidemiological data into stock or ecosystem assessment approaches and models is a step that has begun to be taken in the field of fisheries and marine management ("next generation forecasting in marine ecosystems"), as it provides valuable insight (Lloret et al., 2012; Bolin et al., 2021). In this way, the assessment approaches are more comprehensive, as they are also based on information

related to the physiology of the fish rather than information and projections on distribution alone (Bolin et al., 2021). In this sense, the data presented in this manuscript could be useful in an ecosystem-based management framework and in all those new assessment methods that can contribute to sustainable catches.

Focusing on the current management, the need to reduce fishing pressure from sardine stocks, especially in their Mediterranean distribution, is clear since overexploitation continues to be evident (FAO, 2022). Despite thinking of decreasing fishing quotas as the first measure due to the obvious effects of overfishing in reducing genetic diversity (Pinsky & Palumbi, 2014), body sizes and age/length at maturity (Stergiou, 2002), etc., there are indirect control regulations on harvesting fish stocks such as the minimum landing size (MLS), seasonal and temporal closures (Tosunoğlu et al., 2023) or the implementation of closure of areas. Surprisingly, current MLS for sardine throughout its whole European distribution, involving both Atlantic and Mediterranean waters, was established in a total length of ≥11 cm (EU (2019), REGULATION EU 2019/1241). Throughout this thesis we have discussed the variability among the stocks evaluated, with large differences, especially, between Atlantic and Mediterranean individuals in terms of relative condition and size. In fact, all Atlantic sardine individuals presented a size over 13.5 cm, among which some immature individuals were found. In this sense, one can also speak of maximum catch sizes, as the body condition and size of the sardine is dependent on age (Brosset, 2016; Van Beveren et al., 2014). In this context, maternal effects are linked to sardine's reproductive potential and, therefore, the success of the recruitment, since egg quantity is positively linked to fish size, while the egg quality was positively related to lipid reserves (Brosset et al., 2016). Thus, studying the sizes in which reproduction is maximized according to the stock could be advantageous to avoid the capture of a great part of the largest females.

On the other hand, the different reproductive phenology among stocks verified is reason enough to affirm that the closed periods during the reproductive season must be established according to the features of each stock. Contrary to what happens with the MLS, there are different purse-seiners closed seasons depending on the area (e.g., Mediterranean: in Northern Catalan Coast [GSA 6], from December to January; in the Aegean [GSA 22], closure from mid-December to the end of February (Tsikliras & Koutrakis, (2013); in the Northern Adriatic [GSA 17], the closure period for the Italian pelagic trawlers is in August, while the closure is from mid-December to mid-January in Croatia (Farrugio & Soldo, 2014); and in the Strait of Sicily [GSA 16] occurs from December to January (Basilone et al., 2021)), but in many cases, these closed seasons do not completely overlap with the spawning capable-actively spawning phases, which can be problematic for the recruitment and the health of the stocks. This occurs, for example, in the Catalan Coast [GSA 6], as we registered

that the 31 % of the individuals at active spawning or about to start spawning were captured in October and November. In the Northern Adriatic, a closed season in August does not protect the spawning peak of sardine in the area, from November to February.

In addition to the measures discussed above, to mitigate or avoid overexploitation and the poor state of the stocks, it is essential to consider the genetic population units shaped by a set of unique environmental conditions and intrinsic particularities. With the SECTION I of this thesis (**Chapter 1**), we could observe that the management of this resource presents some inconsistencies based on the potential genetic stocks that have been described to date. In this regard, it has been seen that a large number of management units and components to diagnose sardine's state of health have been determined in the Mediterranean, leaving out the most plausible genetic structure. A clear example are the annual reports generated by the Working Group on Stock Assessment of Small Pelagic Species in the framework of the General Fisheries Commission for the Mediterranean (GFCM), which evaluate sardine status considering the geographical subareas (GSAs) as differentiated stocks (see, for example, sardine report from 2022 (FAO/GFCM, 2022)). This overestimation can produce large assessment errors as the biases in estimating fishing mortality and yield, misinterpreting the dynamics of areas where the species has been historically depleted (e.g., the case of the Gulf of Lion (GSA 7) and the Catalan Coast (GSA 6), in which the focus was on the drop in sardine abundance of the former stock despite the genetic homogeneity with respect to individuals from the Catalan Coast). Moreover, this could promote the transference of that fishing pressure to another genetic stock that is actually in worse condition (Caballero-Huertas et al., 2022a). In fact, the lowest condition, including relative condition and indirect energy estimates, was observed in the Northern Spain stock, consisting of samples from the Catalan Coast. Despite the ecological explanations provided in **Chapter 4** (i.e., high temperatures, plankton shift, etc.), an erroneous management could have contributed to the poor state of health of these individuals.

However, up to now everything seems to indicate that Adriatic-Ionian (Tinti et al., 2002; Kasapidis et al., 2012) and Alboran (Ramon & Castro, 1997; Kasapidis et al., 2012) are the only stocks in which there seem to be clear genetic differences compared to the rest of the Mediterranean. Some doubts emerge regarding the Aegean stock and there are multiple hypotheses in the Iberian Peninsula. In this sense, the apparent connectivity shown in the SECTION II between the Atlantic and the Alboran throughout the spawning season (migration of the Atlantic individuals towards the Alboran), reinforced by previous studies in which a genetic similarity of the stocks was recorded (Coll & Bellido, 2019; Antoniou et al., in press), makes one think that the management and the assessment of such stocks

should take this fact into account. If a study of the condition, sizes, gonad maturation, etc., was carried out throughout the autumn-winter in the Alboran, the results may be masked by the characteristics of the Atlantic individuals, which present an apparently better health status. In this sense, protecting connectivity promotes successful fisheries conservation (Fontoura et al., 2022), as it plays an important role in local and metapopulation dynamics, community structure, and the resilience and avoidance of the decline of fish populations to human exploitation (Muñoz et al., 2015; García-Lafuente et al., 2021). In this concrete case, connected stocks are administratively unconnected so that we highlight the necessity to manage the area under a collaborative approach among countries and considered by international fisheries organisms. Taking all the above into account, we can affirm that the excessive particularisation of Mediterranean stocks into management units, which do not consider the potential genetic structure or the migration and connectivity of a pelagic species with often different feeding and reproduction environments (McBride et al., 2015), does not seem ideal for managing a species that has shown great declines in recent decades. Thus, our proposal is to assess and manage the southern area of Portugal - south-western Spain (Southern European Stock, ICES Subareas 27.8.c and 27.9.a, fished by Spain and Portugal (ICES, 2017)) as a co-shared stock with the GFCM, considering combined GSAs 1, 2 and 3. This would even be potentially useful for other species in which connectivity may play a fundamental role, as the European hake and the blackspot seabream (García-Lafuente et al., 2021).

On the other hand, in this thesis we have detected that the delimitation of Atlantic management units is not ideally established either. The most appropriate management approach in African waters should be based on establishing a Northern and Southern African stocks, delimited by the Bay of Agadir (30° 48′ N), although this barrier to gene flow could represent the true boundary between two great stocks at a global Atlantic level in the sardine distribution range: a Northern and a Southern Atlantic stocks (Caballero-Huertas et al., 2022a). Moreover, it was proposed a differentiated stock consisting of Azores and Madeira, similar from a genetic perspective (Kasapidis et al., 2012). In EU Atlantic waters, the two sardine stocks (Northern stock (ICES Subareas 27.7 and 27.8.a, b, d) and Southern stock (ICES Subareas 27.8.c and 27.9.a)) are considered separately, although due to the existing doubts regarding the genetic stocks along the Iberian Coast, it is unknown whether they belong to the same genetic pool, despite the fact that current studies point to the subareas that comprise the Bay of Biscay (ICES Subareas 27.8.a, b, c, d) as a single distinct unit (Caballero-Huertas et al., 2022a). In this regard, underestimating genetic population differentiation in harvest management could hinder genetic diversity recovery, as when an entire species is considered one stock, there are no potential refugia (Pinsky & Palumbi,

2014). Likewise, and following the line of conservation of the state of health of the stocks, the review of the available genetic structure information and the development of a metapopulation scheme of the sardine in its distribution is a first step to a further implementation of approaches, such as evolutionary impact assessments and genetic monitoring, to predict long-term sustainability of the stocks based on genetic changes driven by environmental pressures in temporal series (Allendorf et al., 2014).

Finally, in addition to protection measures for the sardine stocks under study or any others, the implications of the parasitic characterisation with respect to the revaluation of sardines as a consumer product must be mentioned. First of all, and using one of the most effective techniques in terms of parasite detection (i.e., UV-press), we observed a low presence of nematodes in all the stocks (from 0 in the Alboran and Aegean to a prevalence of 7.6 % and mean intensity 1.70 in the Northern Adriatic), all of them located in the viscera. Of the nematodes found and identified by direct sequencing, the most abundant was *Hysterothylacium aduncum*, with low risk for humans after ingestion. Two species of *Anisakis* were observed in two of the stocks, with a prevalence of 0.8 % of *Anisakis pegreffii* in the Atlantic and the Northern Adriatic, and 1.7 % of *Anisakis simplex* (s.s.) in the Atlantic. These values are lower than other small pelagic species, as anchovy (*Engraulis encrasicolus*) (Cipriani et al., 2018), which presents higher levels of parasitism in all locations for *H. aduncum* and *A. pegreffii* except in Alboran, also without the presence of ascaridoids. In addition, in the anchovy the *Anisakis* genus was also found in a low percentage (4.2 %) in the flesh. Due to these low levels of nematodes in sardine individuals and its absence in the edible part (i.e., flesh), we can consider this species as a safe fish to be eaten, which also has multiple beneficial nutritional properties for humans (omega-3 fatty acids and essential amino acids) with low greenhouse gas emissions per unit catch (Šimat et al., 2020; Robinson et al., 2022), as well as a low content of heavy metals (Sofoulaki et al., 2019). In **Chapter 5**, it was highlighted the clear link between the reproductive stage of the sardine and parasitisation, being active spawners the stage in which more ascaridoids were found. Although these are low parasite loads, the fact of relating the reproductive season of sardines, which must be protected by closed seasons or low catches, with the season of highest prevalence of nematodes could be key for the demand of the consumer. Thus, when the sardine is in stages other than reproductive, with higher lipid levels, better condition and state of health, fewer ascaridoids, etc. (spring and summer months), the value of the fishing resource should be increased, covering the needs of the sector in those less productive periods for the recovery of the species. Therefore, and given the attractiveness of this fish for consumption, it could allow a revaluation, thus making the drop in fishing quotas sustainable for sardine fisheries, as the most interesting approach for fishermen and

the sector (as well as the species) would be to maximize profitability by minimizing extraction.

3. Limitations and future research perspectives

Due to the difficulty and limitations in collecting, transporting, and storing samples throughout such a large distribution range, seasonal sampling was carried out in each of the subareas under study throughout a concrete time period (2019 to 2021). This was not a problem for the development of our work and for obtaining a current global vision of the differences among stocks (in addition to deciphering physiological aspects applicable to sardines as a species), although a monthly sampling over multiple years would have allowed a more detailed study of the health status and the influencing factors in each of the subareas.

Moreover, despite the fact that freezing does not affect condition status measurements (Brosset et al., 2015a), having fresh samples would have allowed us to ideally perform gonadal histology in all the chapters to provide even more information on the fecundity of the species and its relationship with somatic condition and the energy allocated to reproduction. Although numerous investigations have verified that a greater size and better condition usually revert to a greater reproductive potential (basically, greater fecundity and quality of the eggs and, consequently, greater recruitment and fishing productivity), many of them also demonstrate the existence of temporal and regional variations in these reproductive parameters (see, for example, Murua & Saborido-Rey, 2003 or Alonso-Fernández & Saborido-Rey, 2011). In this sense, it should be noted that there are areas in which there are no data on the fecundity of sardines (as in the North-western Mediterranean), and that this information is very relevant to be able to determine and implement a minimum optimal capture size, since small increases in size can represent exponential increases in the number of eggs laid (Barneche et al., 2018). At present, our group is starting the 2021 Ecological Transition and Digital Transition project 'SARDTEMP', in which this study is being carried out on the northwest coast of the Iberian Peninsula.

In the same way, it would have been interesting to relate the data obtained on the state of health (condition, parasite load, etc.) with an analysis of trophic ecology through, for example, the study of stomach content and stable isotopes. In this line, including the analysis of the lipid profile of sardines (both muscle and liver) would be of great help to understand the variations of the diet and its assimilation according to spatial variability, the season, and the reproductive cycle. In addition, the lipid profile of the liver would help us to complete the study of the role of the liver in sardine and the differences among stocks

(**Chapter 4**), and that of the muscle would contribute as well to the revaluation of sardines as a consumer product. With the information acquired (physiological and environmental variables) combined with other potential covariates already mentioned here, we could develop a model that relates them and which could provide statistical values that give more weight to some of the conclusions, in some cases, obtained from characterisations of the areas and their relationship with sardine's quantitative and qualitative indices (e.g., **Chapters 3** and **4**). However, this needs to be carefully studied, although we look forward to testing it in the near future.

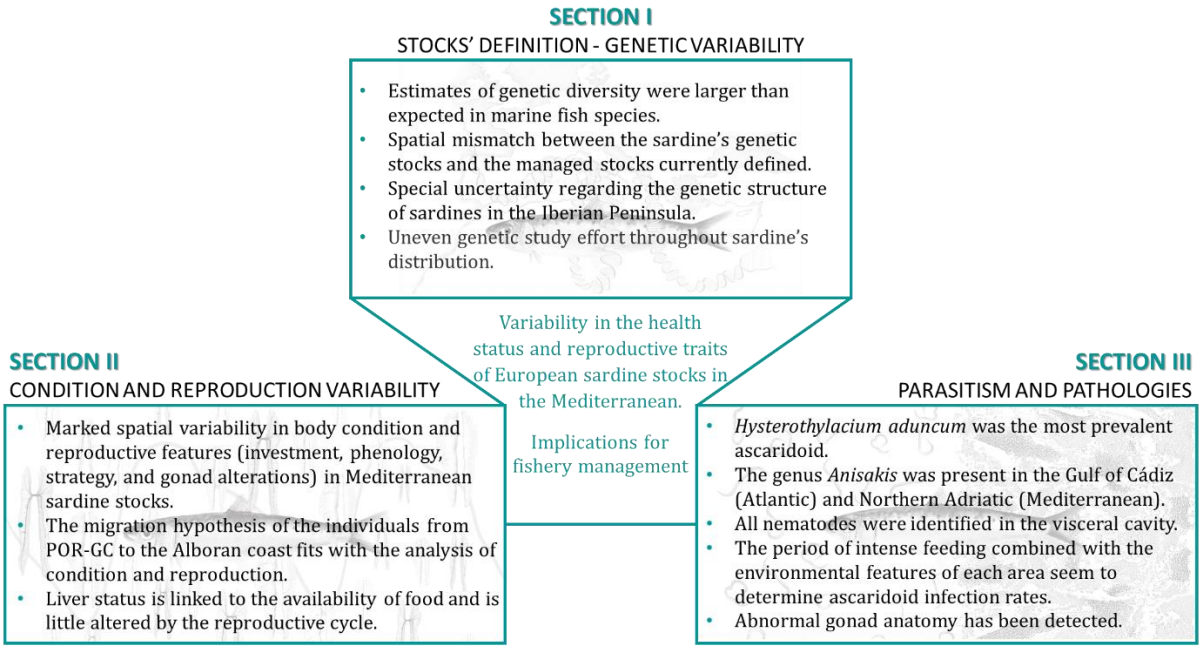
Going back to our results, Atlantic spawners would coincide in space and time with the autochthonous individuals of Alboran that are starting the gonadal developmental cycle. This would explain the simultaneous discovery of sardines in two stages of gonadal development in the same place and time. Previous studies in sardines detected genetic connectivity based on a distance isolation model between populations in the Atlantic and the Mediterranean Sea (González & Zardoya, 2007). In this line, it is intended to analyse genetically the existing population units in the Atlantic-Mediterranean transition and, in this way, to confirm the connectivity making use of our samples, which will be supported by our condition and reproduction data. This analysis must be carried out on a monthly basis, with special emphasis on the reproductive season, since in this way we will be able to confirm exactly when we find individuals from the Atlantic in the Mediterranean (Alboran) area and, therefore, to know with genetic accuracy when this migration has occurred. The results obtained will allow us to strengthen our argument for changes in management units to be considered towards a shared scenario.

In addition, the study of condition, reproduction and ascaridoid nematodes load that we have carried out has to be molecularly contextualized in order not only to obtain our own results regarding the population structure of the sardine, but also to find answers to the variability in the health status within and among the different stocks. In this way, a new study unravelling genetic units may complement the existing ones reviewed in **Chapter 1**, paying special attention to the areas in which there are more doubts, among which the Iberian Peninsula stands out. On the other hand, individuals in different states of condition (combination of Kn with tissue fat content data) are being incorporated to this experimental design, considering the same gonadal stage to analyse the genetic particularities of individuals in good state of condition from all the stocks. Thus, the analysis of the population structure is being carried out in combination with the detection of possible genomic regions susceptible to selection processes, related to the phenotypic variability in the state of condition and environmental factors (e.g., temperature). With this purpose, RAD sequencing is being used, which is based on determining the variability of the genome of

individuals by comparing genome fragments generated by restriction enzymes (RAD tags) (Baird et al., 2008).

Moreover, traits influencing the distribution of genetic diversity has major ecological and evolutionary implications for host-parasite interactions (Mazé-Guilmo et al., 2016). Thus, the study of the genetic variability of the ascaridoids present in sardine individuals could give us information about the very genetic structure of the sardine stocks, data that could be overlapped with the RADSeq results obtained from fish DNA. In fact, population genetics of the fish host can detect changes over an evolutionary timescale, providing indications on the cohesive action of gene flow, but parasites are more suitable biomarkers when considering fish stocks over smaller temporal and spatial scales, hence giving information of fish movements over their lifespan (Mattiucci et al., 2015), which is key in pelagic fish as highly dispersal species. Thus, we propose to carry out a similar study to the developed in the review by Baldwin et al. (2012) in Pacific sardine (*Sardinops sagax*) with our own data of sardine morphometrics and condition, fish genetics, parasite genetics, and parasites as biological tags to resolve fish stock structure.

Final conclusions



Box 1. Outline of the main conclusions derived from this Doctoral Thesis. The connection between the three sections and the main findings presented in each of them are shown.

The main conclusions (summed up in Figure 1) derived from this thesis are the following:

- There is a spatial mismatch between the sardine's genetic stocks and the managed stocks currently defined. Management and assessment units are more abundant than the apparent biological units in the Mediterranean basin, the African Coast, or the Azores-Madeira, which are considered different stocks despite their genetic similarities. The opposite occurs in areas of the Atlantic distribution, the Bay of Biscay, not considered a different stock in the current management model.
- Uneven study effort along sardine's distribution regarding the genetic population structure was detected. Genetic information from the Black Sea, the Southern Ionian Sea and the Levantine Sea, as well as from the northern and the southernmost Atlantic distribution is scarce or non-existent.
- The study of condition should include the energy content evaluation based on lipid estimations and not only indices based on length and weight (e.g., Kn).
- Condition parameters, nematode infection rates, and reproductive features are interrelated, and depend on key environmental variables, especially, temperature/temperature variations, productivity (closely linked to proximity to rivers, wind, the upwelling of deep waters, the physical barriers of the coast, etc.),

and those characteristics of the history of life of the stocks that have been shaped through the generations and that are linked to potential genetic differences.

- Marked spatial variability in body condition, ascaridoid load and species, and reproductive features (investment, phenology, strategy, and gonad alterations) was observed in the Mediterranean sardine stocks and considering individuals from the Atlantic waters throughout the seasons. This matches the larger-than-expected estimates of genetic diversity.
- The accessibility to the resources in the environment modulates the reproductive strategy of European sardine. Therefore, the degree of 'capital breeding' is linked to the available resources throughout the annual cycle, as identified in the Northern Adriatic, in which sardines presented a uniform condition in an environment with available feeding all year round, with greater dependence of reproduction on immediate reserves.
- The size of the liver is linked to the stock, probably due to the availability of food (i.e., large livers in highly productive Northern Adriatic), and is little altered by the reproductive cycle, unlike tissue fat content or mesenteric fat, which are directly related to the gonad developmental phases.
- There is uncertainty regarding the genetic structure of sardines in the Iberian Peninsula, which converges with marked differences in condition and reproductive phenology of the three Iberian stocks studied (Gulf of Cádiz and Alboran Sea, with better condition, and Northern Spain (Catalan Coast), with the worst condition observed in this thesis).
- The apparent genetic similarities between individuals from the Gulf of Cádiz (Atlantic) and those from Alboran (Mediterranean) may be due to the connectivity between these two stocks, which fits with the hypothesis of the established migration of individuals from the Gulf of Cádiz to the Alboran coast through the annual analysis of the condition and reproductive stages on one side and the other of the Strait of Gibraltar. This migration could be triggered by a drop in local temperature in the Alboran, helped by the inflow of Atlantic waters in the Mediterranean, and other oceanographic conditions that favour the generation of nursery grounds.
- Three nematode species were identified in the visceral cavity of sardine throughout three (Gulf of Cádiz, Northern Spain, and Northern Adriatic) of the five studied subareas (absence of parasitisation in the Alboran and in the Aegean), being

Hysterothylacium aduncum the most found, although the genus *Anisakis* was present in the Gulf of Cádiz (Atlantic) and the Northern Adriatic (Mediterranean), results to be considered ecologically and commercially.

- The reproductive phenology and, therefore, the moment in which the intense feeding period occurs, combined with the environmental features of each area, seem to be behind the ascaridoid infection rates. Non-infected Alboran and Aegean sardine stocks continue to be reproductively active in spring, with an intensive feeding period close to autumn-winter. In these months, the main zooplanktonic bloom (including copepods, the principal ascaridoid carriers) would not have taken place yet, in addition to the fact that water stratification and temperature would be lower, conditions that do not favour the cycle of ascaridoids or their transmission between hosts.
- The revaluation of sardines as a fishery product during its most optimal months (spring - summer) due to its better condition and lipid content and its low or null presence of nematodes could be considered in a scenario of low catches and a longer closed season during its reproductive period (autumn - winter).
- Abnormal gonad anatomy and architecture has been detected in Mediterranean sardine stocks, with a highest prevalence in the Northern Spain (Catalan Coast) (2 %). However, all the subunits presented functionality, although the cause remains to be determined.

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Supplementary materials

SECTION II

Chapter 2

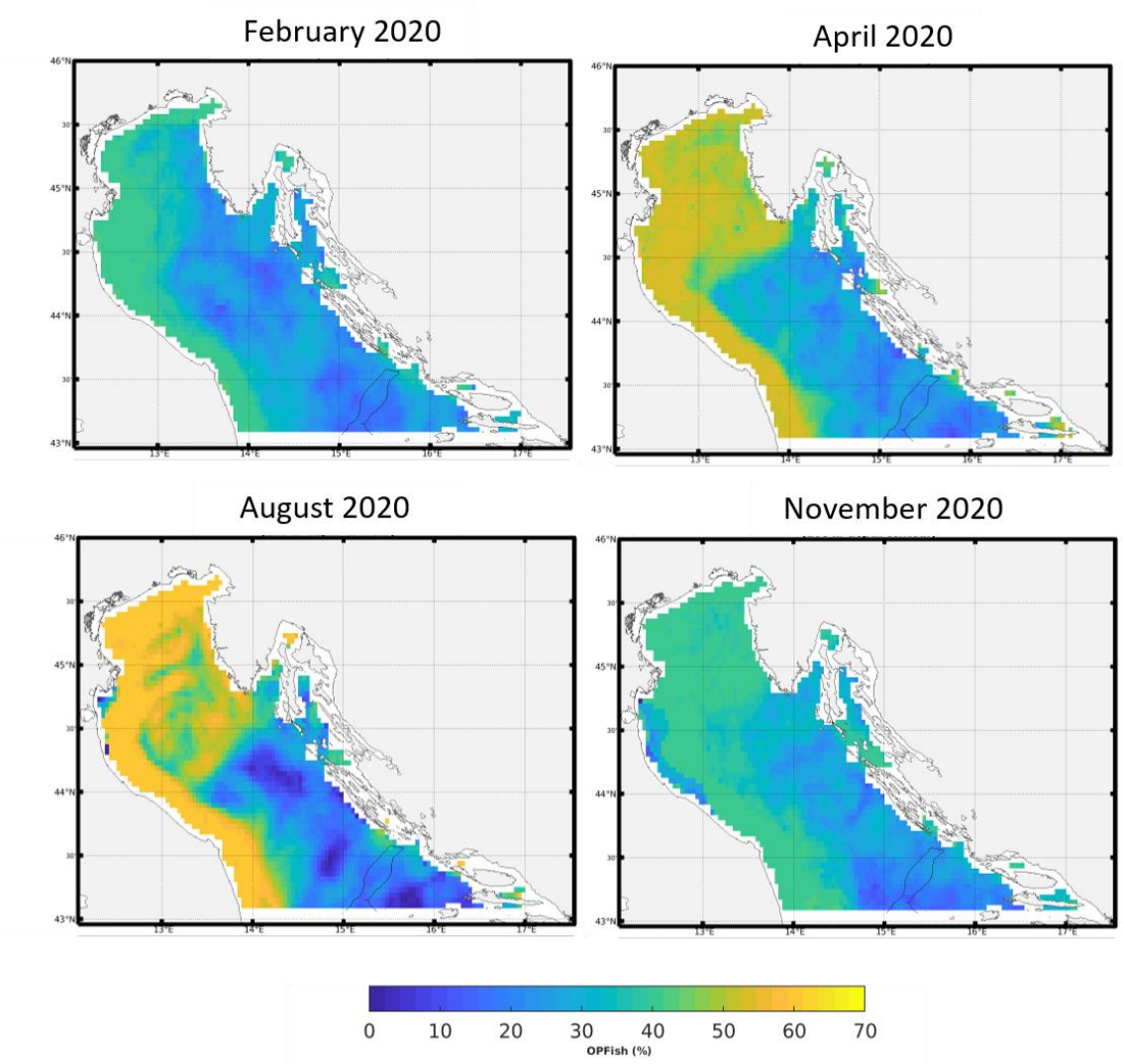

Figure S2.1. Example of the available portion of productivity for the fish (OPFish; %) in months of the year 2020 in the study area (Gulf of Trieste).

Chapter 3

Table S3.1. Summary of the condition indices (mean values ± SD) analysed in European sardine (*S. pilchardus*) in Southern Portugal-Gulf of Cádiz (POR-GC) and Alboran (Alb) coasts: relative condition index (Kn), gonadosomatic index (GSI), hepatosomatic index (HSI), tissue fat content (%), and mesenteric fat scale. Comparisons by sex. One way-analysis of variance (ANOVA) was applied for numeric variables, and Pearson's Chi-squared test was used for categorical variables (i.e., mesenteric fat).

Variable/Index	Area	N	Season	Mean ± SD		*P*-value
				Male	Female	
Kn	POR-GC	189	**Winter** Feb, March	0.85 ± 0.06		NS
		204	**Spring** May, June	0.98 ± 0.11		
		101	**Summer** Sep	1.21 ± 0.11		
		105	**Autumn** Oct	1.18 ± 0.09		
	Alb	20	**Winter** Feb	0.86 ± 0.05		NS
		208	**Spring** Apr, June	0.96 ± 0.08		
		99	**Summer** Aug	1.10 ± 0.08		
		202	**Autumn** Oct, Nov	1.06 ± 0.07		
GSI (%)	POR-GC	189	**Winter** Feb, March	5.64 ± 2.67		NS
		204	**Spring** May, June	1.93 ± 1.85		
		101	**Summer** Sep	0.88 ± 0.83		
		105	**Autumn** Oct	2.20 ± 1.57		
	Alb	20	**Winter** Feb	9.26 ± 3.32		NS
		208	**Spring** Apr, June	1.62 ± 1.34		
		99	**Summer** Aug	0.27 ± 0.18		
		202	**Autumn** Oct, Nov	2.82 ± 2.10		
HSI (%)	POR-GC	189	**Winter** Feb, March	0.47 ± 0.29		NS
		204	**Spring** May, June	0.61 ± 0.44	0.87 ± 0.49	0.00 ***
		101	**Summer** Sep	0.75 ± 0.34		NS
		-	**Autumn** Oct	-		-
	Alb	20	**Winter** Feb	0.24 ± 0.10		NS

		208	**Spring** Apr, June	0.38 ± 0.27		NS
		99	**Summer** Aug	0.74 ± 0.27		NS
		202	**Autumn** Oct, Nov	0.44 ± 0.17	0.67 ± 0.24	0.00 ***
Tissue fat content (%)	POR-GC	189	**Winter** Feb, March	7.72 ± 2.25		NS
		204	**Spring** May, June	11.34 ± 6.54	15.48 ± 7.14	0.00 ***
		101	**Summer** Sep	22.32 ± 2.98		NS
		105	**Autumn** Oct	19.31 ± 2.15		NS
	Alb	20	**Winter** Feb	8.58 ± 1.88		NS
		208	**Spring** Apr, June	7.44 ± 2.20		NS
		99	**Summer** Aug	16.32 ± 2.14		NS
		202	**Autumn** Oct, Nov	13.87 ± 6.28		NS
Mesenteric fat	POR-GC	189	**Winter** Feb, March	1		NS
		204	**Spring** May, June	3	5	0.00 ***
		101	**Summer** Sep	7		NS
		105	**Autumn** Oct	7		NS
	Alb	20	**Winter** Feb	1		NS
		208	**Spring** Apr, June	2		NS
		99	**Summer** Aug	7		NS
		202	**Autumn** Oct, Nov	6		NS

NS: no significant differences between sexes

(-): Data not recorded

***: p-value < 0.0001

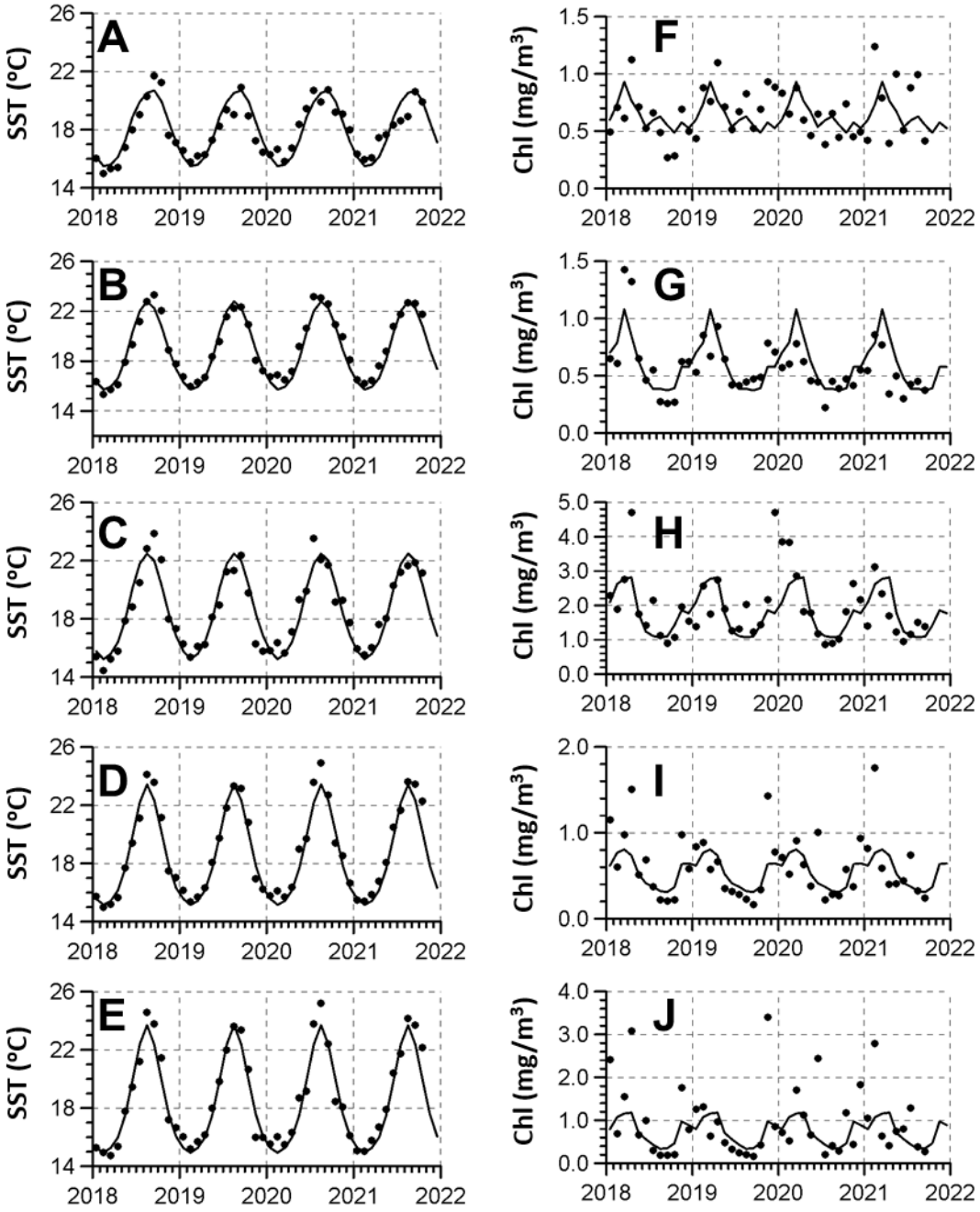

Figure S3.2. Values of SST (ºC) (**A, B, C, D, E**) and Chl concentration (km·h^{-1}) (**F, G, H, I, J**) from one year before the starting of the biological sampling (2018) until 2022 (black dots). A, F: Southern Portugal (POR); B, G: Gulf of Cádiz, Open Sea (GC. O); C, H: Gulf of Cádiz, Coastal area (GC. C); **D, I.** Alboran, Open Sea (Alb. O); **E, J.** Alboran, Coastal area (Alb. C). See Figure 1 for further information regarding the locations. SST solid lines represent the climatological or average values obtained in the period 1981 to 2021, and Chl solid lines indicate the climatological or average values from 1997 to 2021.

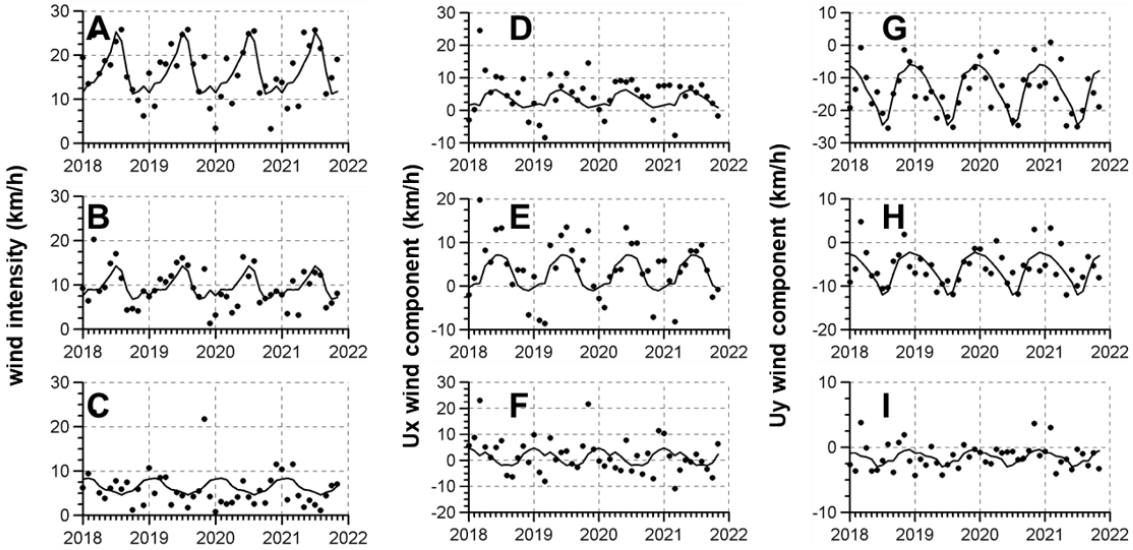

Figure S3.3. Wind intensity (km·h^{-1}) and components Ux (km·h^{-1}) and Uy (km·h^{-1}) of the wind from one year before the starting of the biological sampling (2018) until 2022 (black dots). **A, D, G.** Southern Portugal (POR); **B, E, H.** Gulf of Cádiz (GC. O + GC. C); **C, F, I.** Alboran (Alb. O + Alb. C). See Figure 1 for further information regarding the locations. Solid lines indicate the climatological or average values from 1948 to 2021.

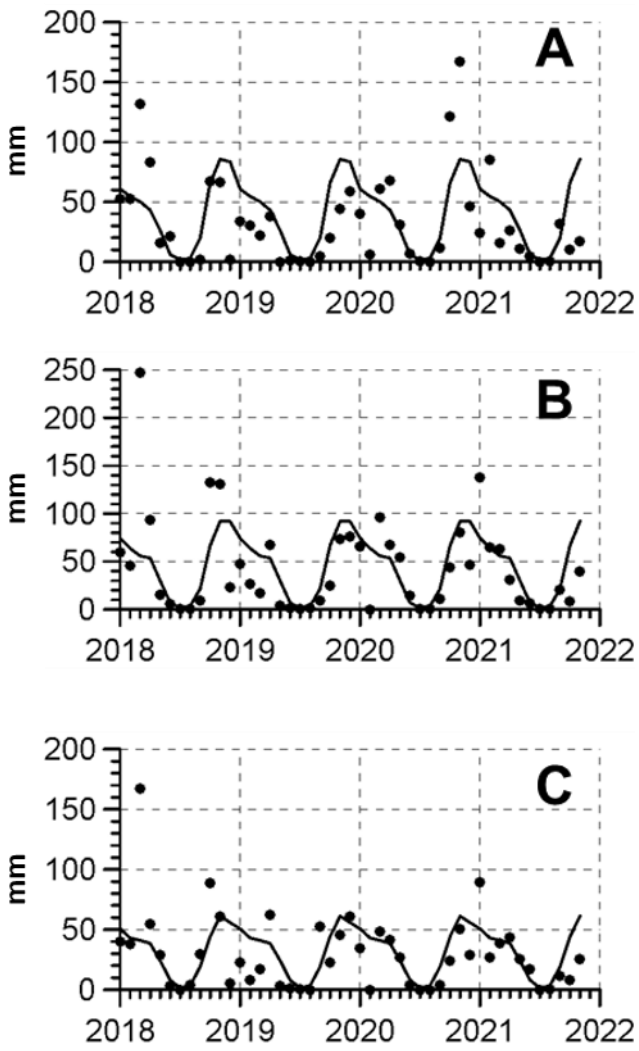

Figure S3.4. Precipitation rates (mm) from one year before the starting of the biological sampling (2018) until 2022 (black dots). **A.** Southern Portugal (POR); **B.** Gulf of Cádiz (GC. O + GC. C); **C.** Alboran (Alb. O + Alb. C). See Figure 1 for further information regarding the locations. Solid lines indicate the climatological or average values from 1948 to 2021.

Chapter 4

Table S4.1. Summary of the sampled individuals of European sardine (*Sardina pilchardus*) by season. N: number of total individuals and the identified by sex (F, female or M, male); Kn: Le Crens's index; HSI: hepatosomatic index. Parentheses include the minimum and the maximum by subarea and season for each parameter.

Area	Season	N		Mean length ± SD (cm)	Kn	Tissue fat content ± SD (%)	Mesenteric fat (median) (#)	HSI ± SD (%)
		F	M					
South Portugal (FAO Division 27.9.a) Atl	Winter Feb, Mar	189		18.372 ± 0.918 (13.5 - 20.4)	0.864 ± 0.063 (0.713 - 1.057)	7.722 ± 2.250 (3.7 - 14.70)	1 (1 - 3)	0.468 ± 0.292 (0.053 - 1.536)
		87	102					
	Spring May, Jun	204		16.859 ± 1.314 (13.9 - 20.1)	0.998 ± 0.113 (0.692 - 1.353)	13.369 ± 7.13 (3.0 - 27.9)	(1 - 7)	0.739 ± 0.482 (0.098 - 2.793)
		100	104					
	Summer Sep	101		17.502 ± 1.185 (14.4 - 21.0)	1.231 ± 0.114 (0.919 - 1.535)	22.325 ± 2.978 (12.2 - 30.25)	(4 - 7)	0.752 ± 0.340 (0.189 - 1.742)
		50	51					
	Autumn Oct	105		18.486 ± 0.815 (16.6 - 22.0)	1.194 ± 0.091 (0.905 - 1.440)	19.309 ± 2.148 (7.9 - 24.95)	7 (2 - 7)	-
		49	56					
Northern Alboran (GSA 1) Med	Winter Feb	20		19.235 ± 0.908 (15.9 - 20.4)	0.868 ± 0.051 (0.794 - 0.976)	8.578 ± 1.883 (4.75 - 11.25)	1 (1 - 2)	0.240 ± 0.097 (0.080 - 0.480)
		7	13					
	Spring Apr, Jun	208		14.958 ± 3.547 (9.1 - 20.4)	0.990 ± 0.092 (0.745 - 1.372)	7.441 ± 2.198 (3.90 - 15.50)	2 (1 - 6)	0.378 ± 0.268 (0.071 - 2.111)
		53	52					
	Summer Aug	99		16.303 ± 0.941 (10.8 - 19.1)	1.129 ± 0.081 (0.922 - 1.442)	16.322 ± 2.144 (6.95 - 20.60)	7 (1 - 7)	0.735 ± 0.266 (0.174 - 1.691)
		44	54					
	Autumn	202					6	0.535 ± 0.229

	Oct, Nov	95	107	16.522 ± 3.149 (11.1 - 23.0)	1.089 ± 0.066 (0.724 - 1.238)	13.874 ± 6.282 (4.05 - 24.65)	(2 - 7)	(0.086 - 1.414)
Northern Spain (GSA 6) Med	Winter Feb, Mar	233	102 131	13.427 ± 1.083 (10.8 - 17.3)	0.857 ± 0.079 (0.673 - 1.293)	5.515 ± 1.641 (2.15 - 11.15)	1 (1 - 4)	0.400 ± 0.256 (0.040 - 1.488)
	Spring May, Jun	110	68 34	13.144 ± 1.012 (9.5 - 16.0)	1.018 ± 0.052 (0.782 - 1.162)	10.013 ± 3.481 (3.50 - 18.40)	5 (1 - 7)	0.520 ± 0.327 (0.074 - 1.971)
	Summer Jul, Aug	105	60 39	13.635 ± 1.016 (10.9 - 15.4)	1.027 ± 0.067 (0.866 - 1.269)	13.489 ± 3.756 (2.60 - 20.95)	6 (1 - 7)	0.480 ± 0.177 (0.154 - 0.949)
	Autumn Oct, Nov, Dec	204	91 113	14.627 ± 1.068 (11.7 - 17.6)	0.981 ± 0.096 (0.794 - 1.968)	11.150 ± 3.274 (4.40 - 17.20)	4 (1 - 7)	0.468 ± 0.192 (0.140 - 1.021)
Northern Adriatic Sea (GSA 17) Med	Winter Jan, Feb	202	114 88	13.811 ± 0.944 (11.2 - 16.0)	0.978 ± 0.109 (0.788 - 1.421)	6.985 ± 2.948 (3.10 - 18.65)	2 (1 - 7)	0.957 ± 0.493 (0.165 - 2.474)
	Spring May, Jun	200	108 92	12.775 ± 0.586 (11.0 - 14.5)	1.065 ± 0.150 (0.834 - 1.711)	12.187 ± 3.349 (4.30 - 22.0)	5 (1 - 7)	0.949 ± 0.456 (0.123 - 2.223)
	Summer Sep	101	63 37	13.391 ± 0.613 (12.3 - 15.6)	1.041 ± 0.069 (0.780 - 1.246)	15.291 ± 1.922 (11.05 - 21.35)	7 (3 - 7)	0.668 ± 0.277 (0.039 - 1.472)
	Autumn Nov, Dec	201	126 75	13.497 ± 0.832 (10.7 - 15.6)	1.014 ± 0.173 (0.786 - 1.764)	8.176 ± 3.994 (3.30 - 16.85)	2 (1 - 7)	0.365 ± 0.261 (0.043 - 2.888)
Aegean Sea (GSA 22) Med	Winter Jan, Feb	216	44 172	12.134 ± 0.865 (10.6 - 16.3)	0.972 ± 0.075 (0.795 - 1.424)	5.142 ± 1.630 (3.10 - 12.60)	1 (1 - 3)	0.432 ± 0.290 (0.056 - 1.927)

Spring Jun	102		12.797 ± 0.530 (11.7 - 14.5)	1.031 ± 0.071 (0.765 - 1.184)	9.800 ± 2.806 (4.35 - 16.85)	5 (1 - 6)	0.570 ± 0.318 (0.073 - 1.369)
	85	17					
Summer Jul	100		13.794 ± 0.577 (12.3 - 15.6)	1.095 ± 0.060 (0.968 - 1.264)	17.509 ± 2.818 (10.85 - 26.20)	6 (2 - 7)	0.750 ± 0.262 (0.071 - 1.342)
	70	30					
Autumn Oct, Nov, Dec	199		13.205 ± 1.078 (10.2 - 15.5)	0.997 ± 0.076 (0.463 - 1.222)	9.666 ± 3.637 (2.05 - 17.90)	3 (1 - 7)	0.355 ± 0.256 (0.041 - 2.213)
	82	11					
		6					

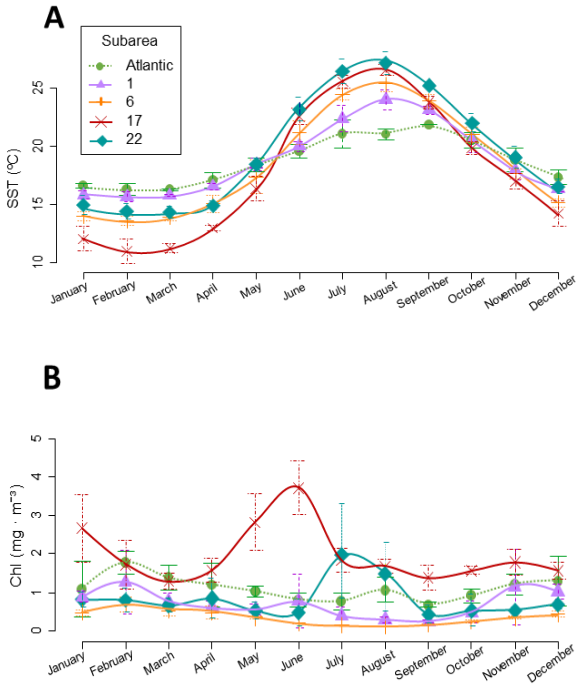

Figure S4.2. Average sea surface temperature (SST, ºC) (**A**) and chlorophyll concentration (Chl, mg · m^{-3}) (**B**) ± standard deviation by subarea in the period 2019 - 2021. Areas defined by the General Fisheries Commission for the Mediterranean (GFCM) as GSA 1 (violet): Northern Alboran Sea (Coast of Málaga), GSA 6 (orange): Northern Spain (Catalan Coast), GSA 17 (red): Northern Adriatic (Gulf of Trieste), and GSA 22 (turquoise): Aegean (Thermaikos Gulf), and the external sampling point in the Atlantic, as FAO division 27.9.a: Portuguese Waters – East (Southern Portugal and Gulf of Cádiz) (green and dotted). NOAA Optimum Interpolation (OI) SST V2 data provided by the NOAA PSL, Boulder, Colorado, USA, from their website at https://psl.noaa.gov, and NASA Goddard Space Flight Center, Ocean Ecology Laboratory, Ocean Biology Processing Group. Sea-Viewing Wide Fieldof-View Sensor (SeaWiFS) Ocean Color Data; In 2018 Reprocessing; NASA OB.DAAC: Greenbelt, MD, USA. Available online: https://oceancolor.gsfc.nasa.gov/, were used for SST and chlorophyll graphs, respectively.

Table S4.3. Summary of the variables/indices comparing subareas of European sardine (*S. pilchardus*) by reproductive stage. L_T: total length (cm); W_T: total weight (g); W_E: eviscerated weight (g); Kn: relative condition index; GSI: gonadosomatic index (%); HSI: hepatosomatic index (%); % Vi: stomach vacuity index (%). Significance values: p-value < 0.05*; < 0.001**; < 0.0001***.

Immature

Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	13.50 ± 0.00	12.19 ± 1.15	12.22 ± 1.32	12.95 ± 0.35	11.37 ± 0.54	79	Welch test	F = 3.07	0.0214*
W_T (g)	15.70 ± 0.00	14.84 ± 4.84	14.29 ± 4.98	17.30 ± 0.42	10.66 ± 1.85	79	Welch test	F = 3.651	0.00906**
W_E (g)	13.60 ± 0.00	13.56 ± 4.54	12.86 ± 4.38	15.60 ± 0.42	9.57 ± 1.65	79	Welch test	F = 3.831	0.00695**
Kn	0.79 ± 0.00	1.07 ± 0.08	1.00 ± 0.06	1.05 ± 0.12	0.97 ± 0.06	79	Kruskal-Wallis	χ^2 = 23.813	0.00008708***
GSI	0.2243 ± 0.0000	0.2834 ± 0.1801	0.1877 ± 0.1511	0.1108 ± 0.0541	0.1784 ± 0.0877	79	Kruskal-Wallis	χ^2 = 9.1255	0.05804 NS
HSI	0.1184 ± 0.0000	0.4477 ± 0.1080	0.5269 ± 0.4054	0.7119 ± 0.0044	0.3310 ± 0.1769	79	Kruskal-Wallis	χ^2 = 8.7205	0.06848 NS
Tissue fat content	6.90 ± 0.00	6.81 ± 2.67	8.78 ± 4.42	15.25 ± 1.70	5.74 ± 1.72	79	Welch test	F = 5.701	0.000469***
Mesenteric fat (median)	1	5	3	6	2	79	Pearson's χ^2 test	χ^2 = 29.479	0.2026 NS
% Vi	0.00	75.00	35.71	0.00	0.00	79	Pearson's χ^2 test	χ^2 = 52.692	0.00000001237***

Developing

Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	17.98 ± 1.04	15.14 ± 2.34	14.55 ± 1.11	13.08 ± 0.75	13.23 ± 0.79	665	Welch test	F = 724.87	2.2e-16***
W_T (g)	57.75 ± 9.54	32.16 ± 18.65	24.33 ± 5.91	18.66 ± 2.66	18.18 ± 3.55	665	Welch test	F = 718.84	2.2e-16***
W_E (g)	51.22 ± 8.54	29.32 ± 16.60	22.28 ± 5.47	16.75 ± 2.43	16.38 ± 3.27	665	Welch test	F = 696.14	2.2e-16***
Kn	1.18 ± 0.12	1.09 ± 0.06	1.00 ± 0.11	1.09 ± 0.13	1.01 ± 0.08	665	Welch test	F = 72.62	2.2e-16***
GSI	1.2048 ± 0.9707	2.1990 ± 0.9159	1.4695 ± 1.093	0.4326 ± 0.1724	2.0885 ± 2.0745	665	Welch test	F = 113.24	2.2e-16***
HSI	0.8209 ± 0.3935	0.6963 ± 0.3733	0.4938 ± 0.1787	1.0299 ± 0.4520	0.4061 ± 0.2906	665	Welch test	F = 59.273	2.2e-16***
Tissue fat content	20.44 ± 3.32	11.02 ± 4.13	12.47 ± 3.33	14.11 ± 2.16	10.20 ± 3.44	665	GLM	z = 3.763	0.000168***
Mesenteric fat (median)	7	6	5	6	4	665	Pearson's χ^2 test	χ^2 = 412.2	2.2e-16***

% Vi	5.38	0.00	15.38	23.77	2.50	665	Pearson's χ^2 test	χ^2 = 159.74	2.2e-16***

Spawning capable

Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	18.44 ± 1.20	19.51 ± 0.86	14.83 ± 1.13	13.41 ± 0.97	13.83 ± 0.94	191	Kruskal-Wallis	χ^2 = 148.71	2.2e-16***
W_T (g)	61.98 ± 13.72	70.23 ± 8.28	25.73 ± 7.06	18.79 ± 3.83	20.76 ± 5.09	191	Welch test	F = 513.92	2.2e-16***
W_E (g)	54.84 ± 11.79	62.07 ± 7.13	23.30 ± 6.54	16.95 ± 3.35	18.69 ± 4.58	191	Welch test	F = 523.4	2.2e-16**
Kn	1.16 ± 0.15	1.10 ± 0.07	0.98 ± 0.08	1.02 ± 0.20	0.99 ± 0.04	191	Welch test	F = 23.217	2.545e-10
GSI	4.2735 ± 1.8342	4.5439 ± 1.4230	3.4604 ± 1.5329	1.9365 ± 1.3709	2.3444 ± 1.6667	191	ANOVA	F = 17.29	4.46e-12 ***
HSI	0.6240 ± 0.2410	0.5077 ± 0.2131	0.4830 ± 0.2141	0.5405 ± 0.5642	0.5032 ± 0.1550	191	Kruskal-Wallis	χ^2 = 4.9247	0.2951 NS
Tissue fat content	17.74 ± 5.89	19.86 ± 2.29	10.93 ± 2.85	8.37 ± 4.22	12.84 ± 3.76	191	Welch test	F = 102.54	2.2e-16***
Mesenteric fat (median)	6	6	4	4	5	191	Pearson's χ^2 test	χ^2 = 126.12	7.788e-16 ***
% Vi	8.33	9.09	22.73	0.00	0.00	191	Pearson's χ^2 test	χ^2 = 69.923	5.091e-12 ***

Actively spawning

Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	17.59 ± 1.45	18.95 ± 0.95	13.65 ± 1.04	13.71 ± 0.89	12.30 ± 1.03	1158	Welch test	F = 1110.3	2.2e-16***
W_T (g)	41.28 ± 10.17	55.15 ± 11.05	17.34 ± 4.73	20.43 ± 4.14	14.65 ± 5.00	1158	Welch test	F = 574.79	2.2e-16***
W_E (g)	36.12 ± 8.83	49.43 ± 9.35	15.57 ± 4.22	17.90 ± 3.40	12.74 ± 4.10	1158	Welch test	F = 613.93	2.2e-16***
Kn	0.88 ± 0.06	0.95 ± 0.09	0.86 ± 0.08	0.99 ± 0.14	0.98 ± 0.08	1158	Welch test	F = 119.03	2.2e-16***
GSI	5.2253 ± 2.4577	4.6119 ± 3.3453	3.3739 ± 1.4386	4.0605 ± 1.7536	5.4905 ± 1.9421	1158	Welch test	F = 57.321	2.2e-16***
HSI	0.4705 ± 0.3196	0.3808 ± 0.3011	0.3773 ± 0.2132	0.6318 ± 0.4655	0.4229 ± 0.2862	1158	Welch test	F = 19.948	5.93e-15 ***
Tissue fat content	7.46 ± 2.27	10.11 ± 5.00	6.18 ± 2.29	7.42 ± 3.43	5.51 ± 2.18	1158	Welch test	F = 40.836	2.2e-16***
Mesenteric fat (median)	1	2	1	2	1	1158	Pearson's χ^2 test	χ^2 = 346.29	2.2e-16***

% Vi	1.20	5.88	41.71	19.32	17.58	1158	Pearson's χ^2 test	χ^2 = 247.5	2.2e-16***

Regressing									
Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	16.22 ± 1.67	18.09 ± 1.09	13.34 ± 1.16	13.28 ± 0.88	13.84 ± 0.86	186	GLM	z = -6.555	5.57e-11 ***
W_T (g)	30.68 ± 7.94	47.32 ± 6.65	18.66 ± 5.35	18.36 ± 4.16	21.82 ± 5.33	186	Welch test	F = 181.01	2.2e-16***
W_E (g)	27.47 ± 7.29	43.36 ± 6.38	16.84 ± 4.92	16.23 ± 3.95	19.62 ± 4.83	186	Welch test	F = 172.11	2.2e-16***
Kn	0.88 ± 0.09	0.98 ± 0.10	0.99 ± 0.09	0.99 ± 0.12	1.04 ± 0.15	186	ANOVA	F = 7.946	0.00000636 ***
GSI	1.3382 ± 0.8301	0.9701 ± 0.5440	0.6750 ± 0.5489	1.4763 ± 0.9229	0.4367 ± 0.1463	186	Welch test	F = 16.492	0.00000003 385***
HSI	0.4464 ± 0.2584	0.4716 ± 0.2791	0.5371 ± 0.2994	0.9856 ± 0.4909	0.8361 ± 0.2406	186	Welch test	F = 9.6597	0.0001329 **
Tissue fat content	6.54 ± 2.40	10.99 ± 4.36	8.70 ± 4.42	8.71 ± 3.91	14.72 ± 6.12	186	Kruskal-Wallis	χ^2 = 22.665	0.0001477 **
Mesenteric fat	1	3	4	3	5	186	Pearson's χ^2 test	χ^2 = 122.82	3.05e-15 ***
% Vi	3.85	6.12	14.55	45.45	0	186	Pearson's χ^2 test	χ^2 = 48.243	0.00000008 877***

Regenerating									
Variable/ Index	Mean ± SD					N	Test	Statistic	p-value
	Atl	GSA 1	GSA 6	GSA 17	GSA 22				
L_T (cm)	17.81 ± 0.77	15.29 ± 1.92	13.67 ± 0.93	12.90 ± 0.57	13.34 ± 0.81	721	Welch test	F = 849.54	2.2e-16***
W_T (g)	54.51 ± 7.00	32.18 ± 11.85	20.29 ± 4.25	17.30 ± 2.40	20.01 ± 4.25	721	Welch test	F = 734.01	2.2e-16***
W_E (g)	48.02 ± 6.32	29.48 ± 10.80	18.44 ± 4.04	15.41 ± 2.16	17.89 ± 3.82	721	Welch test	F = 699.29	2.2e-16***
Kn	1.14 ± 0.11	1.09 ± 0.10	1.02 ± 0.06	1.04 ± 0.13	1.07 ± 0.07	721	Welch test	F = 36.512	2.2e-16***
GSI	0.5290 ± 0.3107	0.4485 ± 0.3741	0.32096 ± 0.2499	0.2929 ± 0.1969	0.3416 ± 0.1754	721	Welch test	F = 15.493	1.236e-11 ***
HSI	0.9417 ± 0.4500	0.6839 ± 0.2844	0.4969 ± 0.2388	0.7369 ± 0.3795	0.6844 ± 0.3049	721	Welch test	F = 23.572	2.2e-16***
Tissue fat content	20.1802 ± 3.5600	12.2360 ± 4.7578	11.9923 ± 3.6743	12.6252 ± 3.8208	14.4915 ± 4.9073	721	GLM	z = 4.649	0.00000333 ***
Mesenteric fat	7	7	6	5	5	721	Pearson's χ^2 test	χ^2 = 239.18	2.2e-16***
% Vi	1.19	12.5	15.00	33.14	3.40	721	Pearson's χ^2 test	χ^2 = 213.18	2.2e-16***

SECTION III

Chapter 5

Table S5.1. Statistic comparison of the body indices of infected vs. non infected European sardines (*Sardina pilchardus*) and the nematode abundance correlation with the indices taking into consideration all sampled individuals by location (Atl: FAO Division 27.9.a Portuguese Waters – East; GSA 6: Northern Spain; GSA 17: Northern Adriatic Sea.

			Presence vs. Absence			**Correlation with abundance**		
Index	Location	Test	Statistic	*df*	P-value	ρ	S	p-value
Length (cm)	Atl	t-test	t = -1.4914	10.989	0.164, NS	0.1139	255190	0.2156, NS
	GSA 6		t = -5.4372	13.059	0.0001 ***	0.1981	2321900	0.0014*
	GSA 17		t = -1.9063	10.616	0.0840, NS	0.1760	308720	0.0443*
Kn	Atl	Welch's t-test	F = 109.16	20.88	9.514e-10***	-0.4069	405170	0.0000*
	GSA 6	Kruskal-Wallis	χ^2 = 1.5003	1	0.2206, NS	0.0773	2671900	0.2152, NS
	GSA 17	Kruskal-Wallis	χ^2 = 1.3491	1	0.2454, NS	-0.0994	411900	0.2587, NS
Tissue fat content (%)	Atl	Kruskal-Wallis	χ^2 = 16.248	1	0.0001 ***	-0.3709	394780	0.0000 ***
	GSA 6	Welch's t-test	F = 5.7981	11.846	0.0333*	0.0889	2638200	0.1537, NS
	GSA 17	Welch's t-test	F = 9.6457	11.921	0.0092*	-0.2229	458190	0.0105*
Mesenteric fat (#)	Atl	Chi-square test	χ^2 = 33.719	3	0.0000***	-0.4689	423010	0.0000*
	GSA 6		χ^2 = 17.358	6	0.0081*	0.0807	2662100	0.1957, NS
	GSA 17		χ^2 = 21.782	6	0.0013*	-0.2594	471880	0.0028*
GSI (%)	Atl	Kruskal-Wallis	χ^2 = 21.824	1	0.0000***	0.4272	164960	0.0000***
	GSA 6		χ^2 = 1.7889	1	0.1811, NS	-0.0824	3026500	0.1888, NS
	GSA 17		χ^2 = 2.4208	1	0.1197, NS	0.1385	322770	0.1147, NS
HSI (%)	Atl	Kruskal-Wallis	χ^2 = 1.215	1	0.2703, NS	-0.114636	130950	0.2847, NS
	GSA 6		χ^2 = 3.7686	1	0.0522, NS	0.1279	2034600	0.0474*
	GSA 17		χ^2 = 5.1009	1	0.0239*	-0.2053	441320	0.0191*

Table S5.2. Statistic comparison of the body indices of infected vs. non infected European sardines (*Sardina pilchardus*) and the nematode abundance correlation with the indices at the principal season of infection for each locality (Atl: FAO Division 27.9.a Portuguese Waters – East (winter); GSA 6: Northern Spain (spring); GSA 17: Northern Adriatic Sea (autumn).

			Presence vs. Absence			**Correlation with abundance**		
Index	Location	Test	Statistic	*df*	P-value	ρ	S	p-value
Length (cm)	Atl	t-test	t = -0.1746	14.433	0.8638, NS	0.0810	4130.9	0.6705, NS
	GSA 6		t = -5.6906	11.674	0.0001 ***	0.3996	42205	0.0004*
	GSA 17		t = -3.2626	9.3494	0.0093*	0.3975	6422.5	0.0110*
Kn	Atl	t-test	t = 0.9456	23.472	0.354, NS	-0.1163	5017.6	0.5407, NS
	GSA 6		F = 0.1834	6.0934	0.6832, NS	0.0355	67801	0.7621, NS
	GSA 17	t-test	t = 2.7559	26.301	0.0105*	-0.1987	12778	0.2191, NS
Tissue fat content (%)	Atl	t-test	t = -1.0407	14.721	0.3148, NS	0.1344	3890.8	0.4788, NS
	GSA 6		t = -1.1377	19.55	0.269, NS	0.0838	64406	0.4745, NS
	GSA 17		t = 1.0835	6.8473	0.3153, NS	-0.2208	13014	0.171, NS
Mesenteric fat (#)	Atl	Chi-square test	χ^2 = 0.3409	1	0.5593, NS	-0.1096	4987.6	0.5643, NS
	GSA 6		χ^2 = 8.4109	5	0.135, NS	0.0002	70286	0.9986, NS
	GSA 17		χ^2 = 10.351	5	0.0658, NS	-0.2459	13281	0.1262, NS
GSI (%)	Atl	t-test	t = -2.2777	17.764	0.0354*	0.3778	2796.6	0.0395*
	GSA 6	Kruskal-Wallis	χ^2 = 1.0508	1	0.3053, NS	0.1337	58495	0.256, NS
	GSA 17	t-test	t = -0.7683	7.8087	0.4649, NS	0.1168	9415	0.473, NS
HSI (%)	Atl	t-test	t = -0.8036	11.946	0.4374, NS	0.1426	3480.9	0.4605, NS
	GSA 6		t = -1.1829	7.1019	0.2749, NS	0.1810	35772	0.1522, NS
	GSA 17		t = 1.5065	7.0285	0.1755, NS	-0.2236	13044	0.1654, NS