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Aquatic Sciences and Engineering aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of aquatic sciences. The journal publishes original research and review articles that are prepared in accordance with the ethical guidelines.

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## Seasonal Zooplankton Distribution and Species Composition in the Eastern Sea of Marmara

Ezgi Türkeri<sup>1</sup> , Melek Isinibilir<sup>1</sup> 

Cite this article as: Türkeri, E., & Isinibilir, M. (2024). Seasonal zooplankton distribution and species composition in the Eastern Sea of Marmara. *Aquatic Sciences and Engineering*, 39(3), 137-147. DOI: <https://doi.org/10.26650/ASE20241433561>

### ABSTRACT

Seasonal changes in the composition and abundance of zooplankton and their responses to environmental changes were investigated at 30 stations in the upper layer and 13 stations in the lower layer (in February, May, July, and December) of the Eastern Marmara Sea in 2016. A total of 44 species/groups (including jellyfish species) were documented in the study area, with 39 identified in the upper layer water and 31 in the lower layer water. The abundances of zooplankton ranged from 29 to 2822 ind.m<sup>-3</sup> in the upper layer and from 6 to 2283 ind.m<sup>-3</sup> in the lower layer. *Acartia clausi*, *Paracalanus parvus*, *Penilia avirostris*, and *Oithona similis* were the dominant species in summer and autumn, whereas *Calanus euxinus*, *Pseudocalanus elongates*, *Oithona nana*, *Oithona davisae* and *Pleopis polyphemoides* were the dominant species in winter and spring. *Aetideus* spp., *Clausocalanus* spp., *Ctenocalanus vanus*, *Oncaea minuta*, *Isopoda*, and *Siphonophora* were observed only in bottom-layer waters. *M. lucens* and *O. davisae* were recorded only in the upper layer in December. In conclusion, our results suggest that zooplankton communities and some species are favorable indicators of the marine environment of the Sea of Marmara.

**Keywords:** Zooplankton, abundance, species diversity, Sea of Marmara

### INTRODUCTION

Marine coastal areas feature greater levels of plankton biodiversity and production, which play an important role in biogeochemical processes. However, climate change and anthropogenic pressures that degrade ecosystems threaten biodiversity and ecological functions. Increasing anthropogenic activities accelerate eutrophication, often leading to irreversible degradation (Tüfekçi et al., 2010; Kemp and Boynton, 2012; Griffith and Gobler, 2020).

Zooplanktons are highly sensitive to physico-chemical parameters and biological factors in marine systems (Isinibilir et al., 2008; Shi et al., 2015). The composition and abundance of zooplankton largely depend on environmental conditions and respond rapidly to environmental changes (Isinibilir et al., 2011). As a result,

zooplankton is considered a biological indicator of environmental water quality. Zooplankton orchestrate marine ecosystems by not only transferring energy from primary producers to higher trophic levels but also regulating phytoplankton production and shaping the pelagic ecosystem (Rissik et al., 2009).

The Marmara Sea is an important transitional basin between the Black Sea and the Mediterranean, connected to the Black Sea via the Bosphorus and to the Aegean Sea via the Dardanelles. The upper layer of the Marmara Sea is influenced by low salinity Black Sea water (18%), while more saline water from the Mediterranean (up to 40%) is found at depths exceeding 20 m (Beşiktepe et al., 1994). Over the last few decades, many factors including excessive nutrients and pollutants, overfishing, the introduction of new species, and climate change have signifi-

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cantly disrupted the Marmara Sea fauna temporally and spatially. These changes have resulted in red tide and mucilage aggregation, the disappearance or sharp decline in population of some species, and a rise in the number of nonindigenous species (Aktan., 2008; İsinibilir et al., 2010; İsinibilir Okyar et al., 2015; Doğan & İsinibilir, 2016; İsinibilir & Yılmaz, 2017, Turkoğlu, 2013). Zooplanktons, the most important link in energy transfer in the seas, were the first to react to these changes (İsinibilir et al., in press).

The main aim of this study is to explore the abundance and fluctuations in species composition of coastal zooplankton communities in the eastern region of the Sea of Marmara. Furthermore, the study seeks to quantify the zooplankton composition in the research area to determine the indicator species composition across environmental conditions as well as to improve existing data by measuring the relationships of indicator species in the research area with environmental parameters.

## MATERIALS AND METHODS

### Sampling design and analysis of samples

Upper layer samples exposed to both the Black Sea and local pollution were collected from 30 stations on the Marmara Sea in February, May, July, and December in 2016. Stations İZ9, M11, İZ4, and MD102 were located offshore where there is less anthropogenic stress, representing the open-sea ecosystem of the Sea of Marmara, although they were still impacted by the top layer flow from the İzmit Bay and the Istanbul Strait. Stations MD3, MD4, İZ8, and MD8 were considered less polluted and represented a transitional zone from the neritic to open-sea environment. G2, KÇ, M12, M13, M14, MD1, MD2, MD3, MD4, MD5, MD6, MD7, MD9, MD10, MD75, MY5 YK1, İZ7, İZ6, İZ5, İZ3, İZ2, and İZ1 were in the coastal waters of the Sea of Marmara where anthropogenic stress is greatest. Samples were collected from the Mediterranean-originated lower layer at 13 stations (MD1, MD3, MD4, MD5, MD6, MD7, MD8, MD75, MD102, M11, İZ9, İZ8, and İZ4) during periods characterized by both thermal stratification and haline stratification.

Zooplankton samples (including jellyfish species) were gathered vertically using a WP2 closing net with a 0.5 m diameter and a

157-mm mesh. Samples were collected from the bottom to the start of the mixing layer and from the interface (18–20 m) to the surface. The salinity, temperature, and dissolved oxygen of the whole water column were measured with an SBE-19 SEACAT CTD (conductivity, temperature, and pressure recorder) system. Chlorophyll-a analyses were conducted at all stations in the upper water layer using the methodology outlined in APHA (2000). The zooplankton samples were preserved on board using a 4% formalin seawater solution. Three 1-ml aliquots were obtained from the sampling bottle with a Stempel pipette; counting and diagnostics were performed in the zooplankton counting chamber and stereobinocular microscope.

### Statistical analysis

The zooplankton community was assessed according to the Shannon index of diversity ( $H'$ ) and the number of species ( $S$ ) as described by Shannon and Weaver (1949). Furthermore, using  $\log(x + 1)$ -transformed abundance data and Primer v. 6 software, multidimensional scaling (MDS) studies of similarity between sampling months were performed based on the Bray–Curtis similarity index (Clarke & Warwick, 1994). Using SPSS v22 software, Spearman's rank-correlation coefficient was calculated to find associations between biotic and abiotic factors.

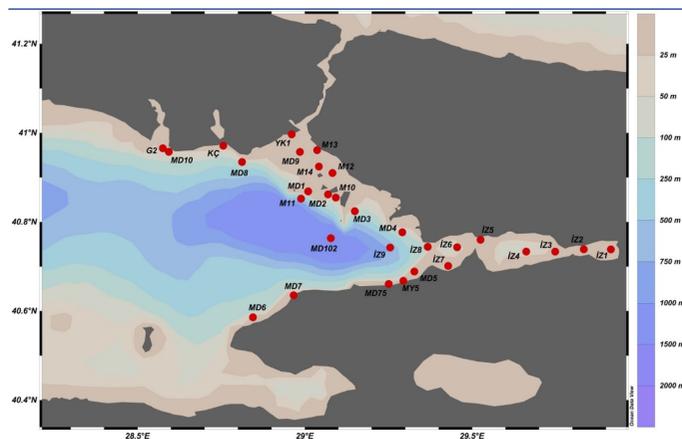
## RESULTS AND DISCUSSION

The two-layered structure is discernible from the profiles of salinity, temperature, and dissolved oxygen (Figure 2). The highest sea surface temperature and chlorophyll-a values were observed in July (25.3°C and 7.7  $\mu\text{g.L}^{-1}$ ), whereas the highest salinity (30.4 ppm) and dissolved oxygen (8.8  $\text{mg.L}^{-1}$ ) values were measured in February (Figure 3). Oxygen values decreased from the surface to the bottom throughout the water column, and values below 2  $\text{mg.L}^{-1}$  were detected deeper than 50 m.

The minimum dissolved oxygen value was recorded in May (4.57  $\text{mg.L}^{-1}$ ). Despite its considerable variability, the distribution of chlorophyll-a exhibited notable spatio-seasonal patterns and a strong correlation with temperature ( $r = 0.526$ ,  $p < 0.01$ ).

Seasonal variations were observed in the abundance and biomass of zooplankton (excluding the dinoflagellate *Noctiluca scintillans*) in the eastern Sea of Marmara (Figure 3). The maximum quantity of zooplankton was observed at station MD6 in December (2822  $\text{ind.m}^{-3}$ ) due to high abundance of *Paracalanus parvus* and *Penilia avirostris* (1210  $\text{ind.m}^{-3}$  and 468  $\text{ind.m}^{-3}$ , respectively). In contrast, the zooplankton biomass peaked at station M11 in May due to *Aurelia aurita* (Figure 3).

A total of 44 species/groups were documented in the study area, with 39 identified in the upper layer water and 31 in the lower layer water (Table 1). The most abundant zooplankton groups across all stations were copepods, cladocerans, meroplankton, appendicularians and jellyfishes. *Aetideus spp.*, *Clausocalanus spp.*, *Ctenocalanus vanus*, *Goniopsyllus rostratus*, *Oncaea minuta*, Iso-poda, and Siphonophora were observed only in bottom-layer waters; *M. lucens* and *O. davisae* were observed only in the upper layer in December.



**Figure 1.** Locations of the sampling stations in the Sea of Marmara.

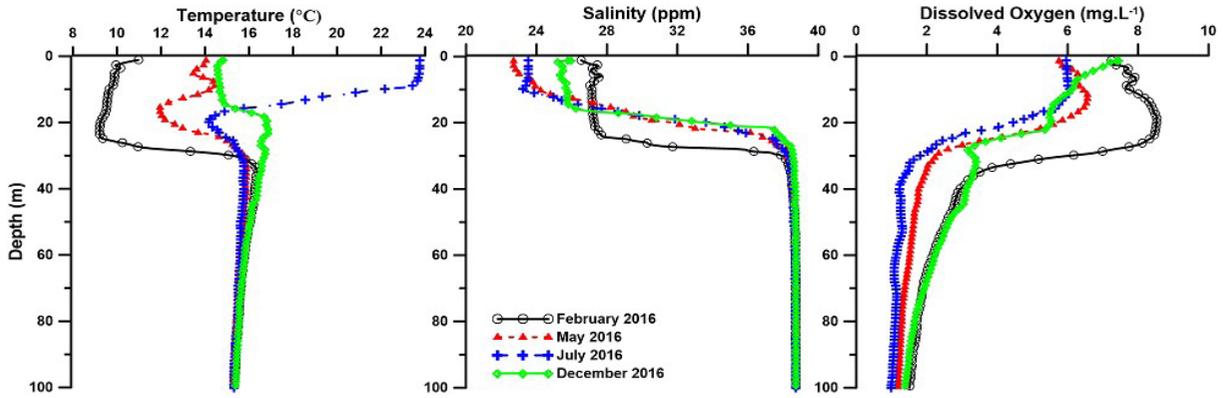


Figure 2. Temperature, salinity and dissolved oxygen profiles in station M11.

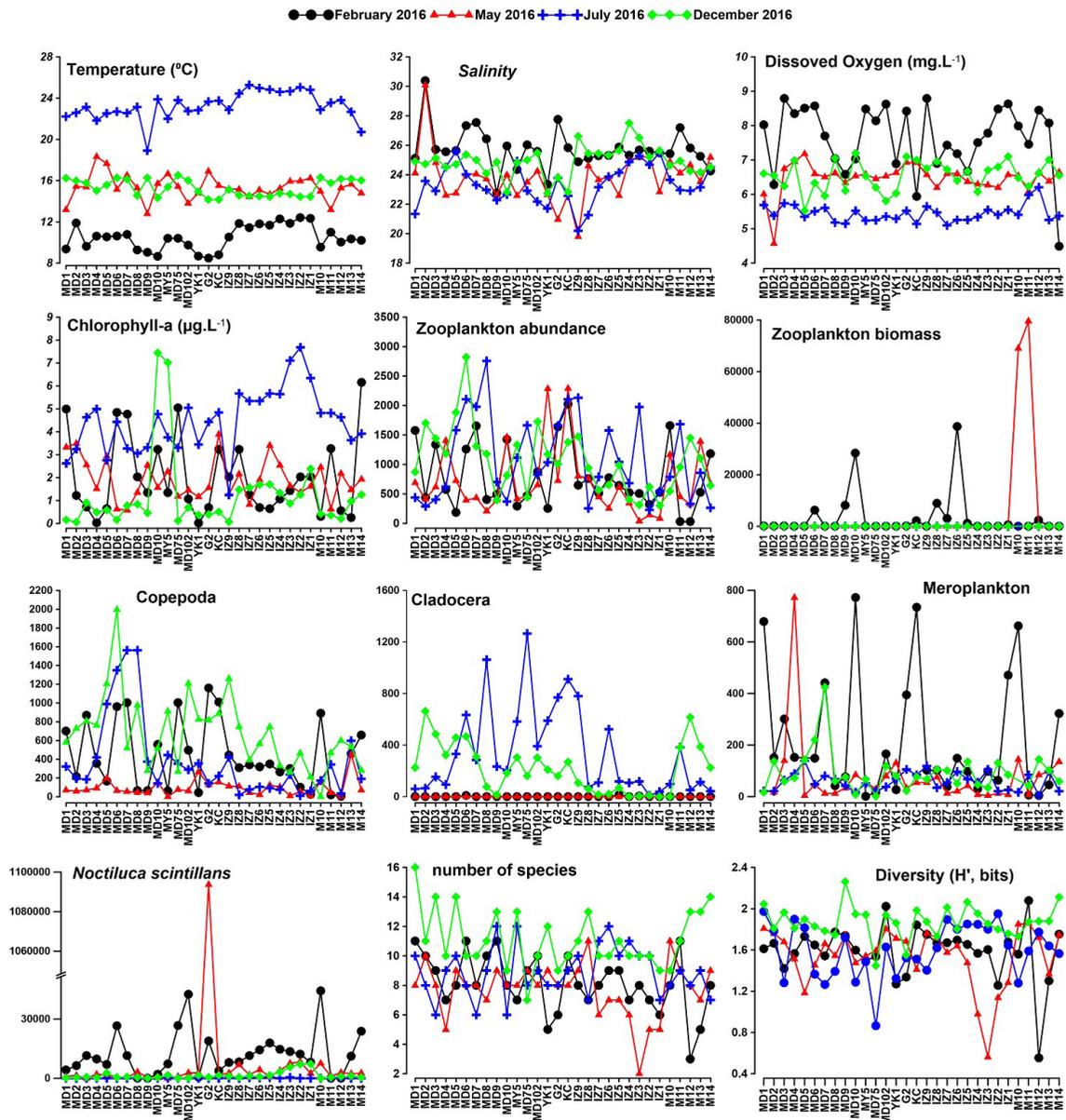


Figure 3. Seasonal fluctuation of temperature, salinity, dissolved oxygen, and chlorophyll-a at the upper layer.

**Table 1.** The total and seasonal mean abundances (ind.m<sup>-3</sup>) of zooplankton species/groups and Noctiluca scintillans in the eastern Sea of Marmara.

	February 2016		May 2016		July 2016		December 2016		Total mean abundance in 2016	
	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
<b>COPEPODA</b>										
<i>Acartia clausi</i> Giesbrecht. 1889	2624.94	285.84	2133.62	166.81	7112.53	745.82	4147.87	239.37	4000.474	359.46
<i>Aetideus</i> spp.	0	0	0	5.27	0	5.27	0	0	0	2.635
<i>Calanus euxinus</i> Hulsemann. 1991	67.01	30.45	0	0	0	0	12.74	2.62	19.937	8.267
<i>Centropages ponticus</i> Karavaev. 1895	15.71	9.26	0	0	0	0	0	0	3.927	2.315
<i>Centropages typicus</i> Kröyer. 1849	51.97	0	11.89	0	8.49	4.37	33.97	7.42	26.58	2.947
<i>Clausocalanus</i> spp.	0	4.25	0	0.31	0	0.24	0	0.24	0	1.26
<i>Ctenocalanus vanus</i> Giesbrecht. 1888	0	12.74	0	5.27	0	5.27	0	12.74	0	9.005
<i>Euterpina acutifrons</i> (Dana. 1847)	82.31	69.78	17.83	8.49	21.23	5.27	433.12	42.48	138.622	31.505
<i>Euchaeta marina</i> (Prestandrea. 1833)	0	2.62	0	0	0	5.27	0	8.49	0	4.095
<i>Metridia lucens</i> Boeck. 1864	0	0	0	0	0	0	29.72	0	7.430	0
<i>Microcalanus pygmaeus</i> (Sars G.O. 1900)	2.62	8.49	0	0	0	0	2.62	4.37	1.310	3.215
<i>Oithona davisae</i> Ferrari & Orsi. 1984	0	0	0	0	0	0	65.19	0	16.297	0
<i>Oithona nana</i> Giesbrecht. 1893	1616.31	211.67	5696.53	1016.85	2005.66	617.09	2057.70	232.40	2844.050	519.502
<i>Oithona similis</i> Claus. 1866	146.07	37.77	325.27	39.28	430.29	95.66	2466.59	509.71	842.055	170.605
<i>Oithona</i> spp.	0	0	0	0	0	2.62	0	0	0.655	0
<i>Oncaea minuta</i> Giesbrecht. 1893	0	17.83	0	25.48	0	17.83	0	25.48	0	21.655
<i>Oncaea</i> spp.	0	2.62	0	0	0	0	0	5.27	0	1.972
<i>Paracalanus panvius</i> (Claus. 1863)	5945.53	550.47	1926.11	308.59	3841.47	652.69	6904.46	245.27	4654.39	439.255
<i>Pseudocalanus elongatus</i> (Boeck. 1865)	0	0	25.48	0	12.74	0	35.47	0	18.422	0
<i>Temora stylifera</i> (Dana. 1849)	0	0	12.74	0	2.62	0	2.62	0	4.495	0
Copepoda nauplii	89.51	27.55	50.96	15.79	56.62	14.76	83.68	13.25	70.192	17.837
<b>CLADOCERA</b>										
<i>Penilia avirostris</i> Dana. 1852	0	0	0	8.49	8980.89	1530.89	6326.71	258.35	3826.90	449.432
<i>Pleopis polyphemoides</i> (Leuckart. 1859)	901.40	63.09	4206.37	15.41	227.88	3.73	80.18	0	1353.957	20.557
<i>Pseudevadne nordmanni</i> Lovén. 1836	8.49	0.24	0	0	12.74	0	0	0	5.307	0.060
<b>APPENDICULARIA</b>										
<i>Oikopleura dioica</i> Fol. 1872	875.30	175.12	976.93	33.20	635.53	135.29	1129.51	85.28	904.317	107.222
<b>CHAETOGNATHA</b>										
<i>Sagitta setosa</i> (Müller. 1847)	74.73	21.91	0	0	0	99.86	167.27	69.52	60.5	47.822

**Table 1.** Continue.

	February 2016		May 2016		July 2016		December 2016		Total mean abundance in 2016	
	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
<b>MEROPLANKTON</b>										
Fish larvae	136.83	8.66	4.25	0	0	0	0	0	35.27	2.165
Fish egg	1524.44	17.66	619.96	13.87	97.86	45.55	31.75	6.55	568.502	20.907
Bivalvia larvae	2505.19	486.75	615.71	82.29	703.47	55.04	1694.80	217.01	1379.792	210.272
Decapoda larvae	2.14	0.20	17.24	6.09	139.79	55.04	7.79	22.21	41.74	20.885
Cirripedia larvae	21.23	0	0	0	0	0	0	0	5.307	0
Polychaeta larvae	230.29	35.41	457.44	28.77	349.61	18.04	256.53	70.22	323.467	38.11
Isopoda larvae	0	0	0	0	0	0	0	0.17	0	0.042
Gastropoda larvae	4.25	0	0	0	0	0	0	0	1.062	0
Reptantia (Brachyura) larvae	2.62	0	0	0	0	0	2.62	0	1.31	0
Pteropoda	0	0	2.62	0	2.62	0	0	0	1.31	0
<b>OTHER GROUPS AND SPECIES</b>										
<i>Aglaura hemistoma</i> Péron & Lesueur. 1810	0	0	0	0.68	0	0	4.76	0.34	1.19	0.255
<i>Aurelia aurita</i> (Linné. 1758)	4.62	3.52	1395.61	2.67	108.85	1.70	991.63	3.40	625.177	2.822
<i>Beroe ovata</i> Chamisso ve Eysenhardt. 1821	14.95	0.59	0.36	4.53	0	0	0.51	0	3.955	1.28
<i>Mnemiopsis leidyi</i> A. Agassiz. 1865	0.25	0	0	0	0	0	0	0	0.062	0
<i>Pleurobrachia pileus</i> (O.F.Müller. 1776)	26.77	7.42	24.29	5.34	0.51	0	0	0	12.892	3.19
Siphonophora (sp.)	0	0.15	0	0	0	0	0	0	0	0.037
<b>Total zooplankton</b>	242339.5	2092.060	51284.98	1793.48	25034.49	4061.96	42510.21	2082.16	89687.463	2978.7395
<i>Noctiluca scintillans</i> (Macartney, Kofoid et Swezy. 1921)	225358.08	0	32763.77	0	283.09	0	15540.40	0	68486.335	0

The abundance of most zooplankton groups displayed distinct seasonal patterns in the eastern Sea of Marmara. In general, copepods and cladocerans were the predominant groups (Table 1), whereas meroplankton became more prominent in coastal regions, eventually dominating the zooplankton community (e.g., stations MD10 and KC in February) (Figure 3). *Acartia clausi* was present year-round, reaching higher densities at station MD7 (913 ind.m<sup>-3</sup>) and İZ9 (904 ind.m<sup>-3</sup>) in July, whereas *P. parvus*, *E. acutifrons*, and *C. euxinus* were the primary members of the copepod community during February and December (Table 1, Figure 4). *Oithona nana*, *Pleopis polyphemoides*, and fine particle filter feeder, *Oikopleura dioica*, dominated the zooplankton community in all seasons, and *Oithona similis* and other fine particle filter feeder *Penilia avirostris* were abundant in July and December. High concentrations of bivalve and polychaeta larvae

were measured in February and May, especially in coastal areas, whereas *Parasagitta setosa* was detected in February, July, and December. *Oithona davisae* was observed only in December and mostly at coastal stations (KC, YK1, MD1, MD3, MD5, M12, M13, M14, MY5, İZ8, İZ7, İZ5, İZ4, İZ3, İZ1).

The highest number of species was observed at MD1 (16 species/groups) in December and the lowest number at İZ3 (3 species/groups) in May and at M12 (3 species/groups) in February. The maximum diversity index was recorded at station MD9 (2.3) in December. The minimum diversity index was 0.6 at station M12 in February and at station İZ3 in May. This decrease in diversity was due to dominance of *P. polyphemoides* and *A. clausi* (Figure 3).

The heterotrophic dinoflagellate *Noctiluca scintillans*, an important component of the net samples in the Sea of Marmara, showed sea-

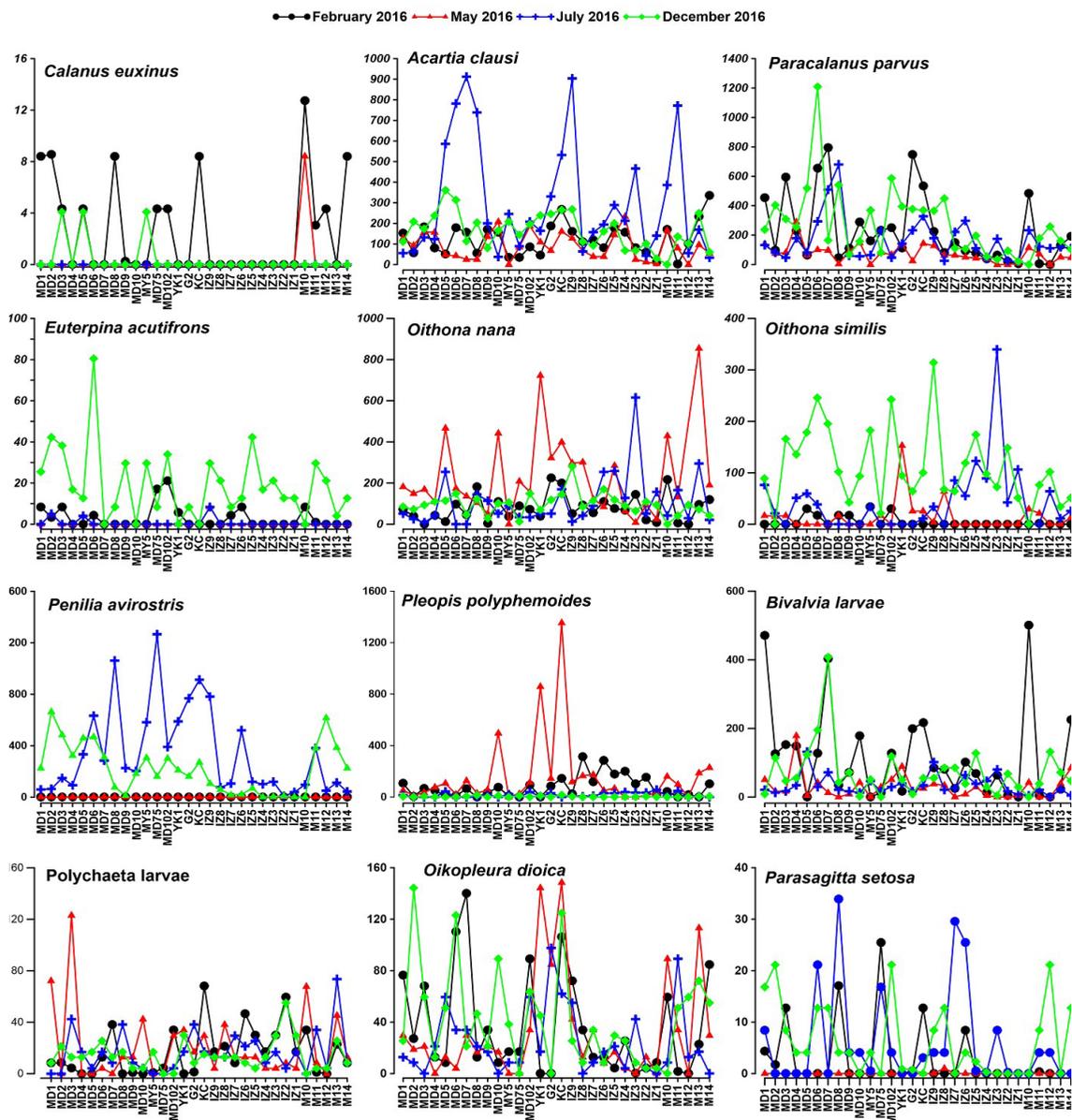


Figure 4. Variations in the abundance (ind.m<sup>-3</sup>) of primary zooplankton species and groups within the upper layer.

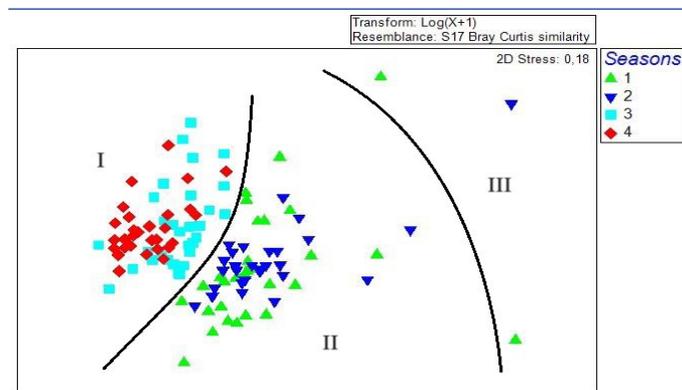
sonal distribution (Figure 2), peaking in February (225358 ind.m<sup>-3</sup>) and reaching its minimum in July (283 ind.m<sup>-3</sup>). *N. scintillans* reached its maximum abundance (1093617 ind.m<sup>-3</sup>) at station G2, located at the entrance of Büyükçekmece Bay. The densities of *N. scintillans* fell to minimum levels in July but increased in December. MDS ordination of combined data across seasons showed that the zooplankton community of the eastern Sea of Marmara had high seasonality (Figure 5). Total zooplankton assemblage exhibited heterogeneity from July to December (Group I) and February to May (Group II). The species predominant from July to December included *Acartia clausi*, *Paracalanus parvus*, *Penilia avirostris*, and *Oithona similis* (Group I); those predominant from February to May community included *Calanus euxinus*, *Pseudocalanus elongates*, *Oithona nana*, *Oithona davisae*, *Pleopis polyphemoides*, polychaeta, and bivalve larvae. Moreover, stations M11 and M12 in February and IZ3 in May (Group III) were distinct from other samplings due to the dominance of *Pleopis polyphemoides*, *Oithona nana*, and *Acartia clausi* as well as low species diversity.

While *P. avirostris* was positively affected by temperature increase ( $r = 0.510, p < 0.01$ ), it was negatively correlated with salinity ( $r = -0.282, p < 0.01$ ) and dissolved oxygen ( $r = -0.400, p < 0.01$ ). Bivalvia larvae were positively affected by salinity ( $r = 0.247, p < 0.01$ ) and dissolved oxygen ( $r = 0.196, p < 0.05$ ) but inversely related to temperature increase ( $r = -0.305, p < 0.01$ ). *A. clausi* was positively affected by temperature ( $r = 0.381, p < 0.05$ ) and negatively correlated with increased salinity ( $r = -0.214, p < 0.05$ ) and dissolved oxygen ( $r = -0.284, p < 0.01$ ).

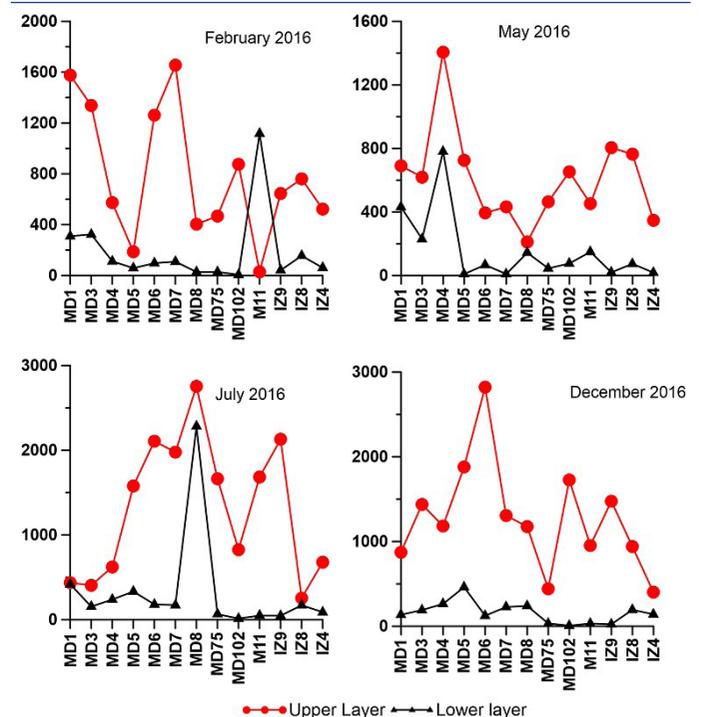
Zooplankton abundance, *P. parvus*, and *P. setosa* positively correlated with temperature ( $r = 0.186, r = 0.194, \text{ and } r = 0.186, p < 0.05$ , respectively), whereas polychaeta larvae ( $r = -0.295, p < 0.05$ ) and *C. euxinus* ( $r = -0.370, p < 0.01$ ) had a negative correlation with it. *E. acutifrons* ( $r = 0.134, p < 0.05$ ) and *O. nana* ( $r = 0.216, p < 0.05$ ) had a positive correlation with dissolved oxygen, whereas *N. scintillans* ( $r = -0.201, p < 0.05$ ) and *P. polyphemoides* ( $r = -0.244, p < 0.01$ ) were inversely related to dissolved oxygen increase. Furthermore, jellyfish abundance was weakly correlated with total zooplankton abundance, *O. similis*, *P. avirostris*, and *P. setosa*, ( $r = -0.107, r = -0.112, r = -0.123, r = -0.103, p < 0.001$ , respectively) but it was positively correlated with *P. parvus* ( $r = 0.151, p < 0.001$ ) and *O. dioica* ( $r = 0.181, p < 0.05$ ).

The upper layer waters generally exhibited a greater zooplankton abundance than the lower layer waters but a lower number of species (Table 1, Figure 6, 7). The maximum average abundance in the upper layer was 2822 – 29 ind. m<sup>-3</sup>, whereas the corresponding value in the lower layer was 462 – 6 ind. m<sup>-3</sup>. The number of species and zooplankton abundance significantly differed between groups; zooplankton were more abundant in the upper layer than in the lower layer. The biggest difference between the lower and upper layers was measured in July and December 2016 when Cladocera dominated the upper layer of Marmara. Species such as *Aetideus* spp., *Clausocalanus* spp., *Ctenocalanus vanus*, *Oncaea minuta*, *Oithona davisae*, Pteropoda, and Siphonophora contribute to the differences in zooplankton communities between the upper and lower layers. The corresponding MDS analysis revealed significant differences between the lower and upper layers (Figure 8).

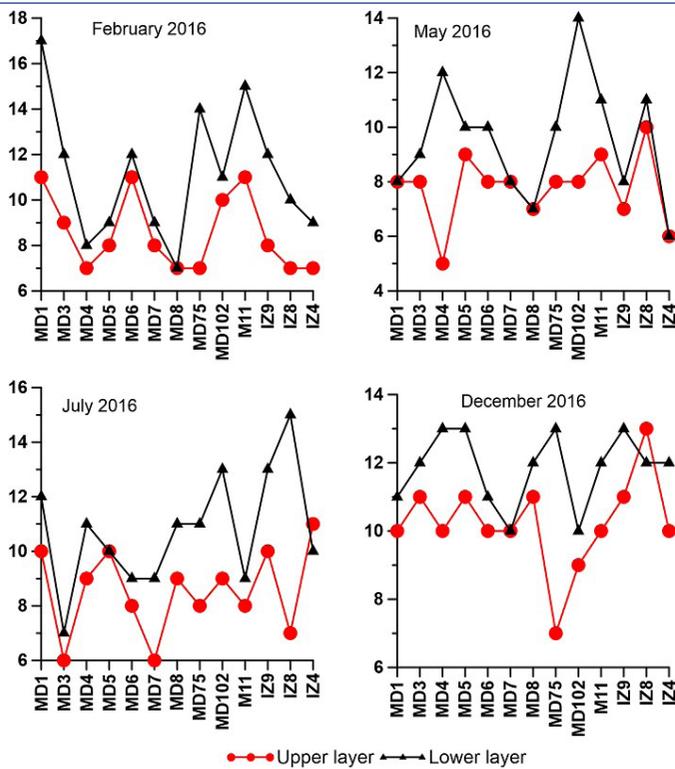
This study identified the major zooplankton species and their seasonal availability in the Marmara Sea. The most widely distributed zooplankton species were also the most prevalent and dominant species such as *Paracalanus parvus*, *Acartia clausi*, *Oithona nana*, *Penilia avirostris*, *Pleopis polyphemoides*, and bivalve larvae. These species are similar to those found in previous studies in the Marmara Sea (Yılmaz et al., 2005; Tarkan et al., 2005; İsinibilir et al., 2011). Notably, *O. davisae*, an invasive species that was first recorded in the Marmara Sea in 2014 (Doğan & İsinibilir, 2016), has expanded its distribution area to the İzmit Bay in the Sea of Marmara. The Sea of Marmara is home to several Mediterranean species, including *Oncaea minuta*, *Aetideus* spp., *Ctenocalanus vanus*, *Clausocalanus* spp., *Euchaeta marina*, and Siphonophore (İsinibilir et al., 2011).



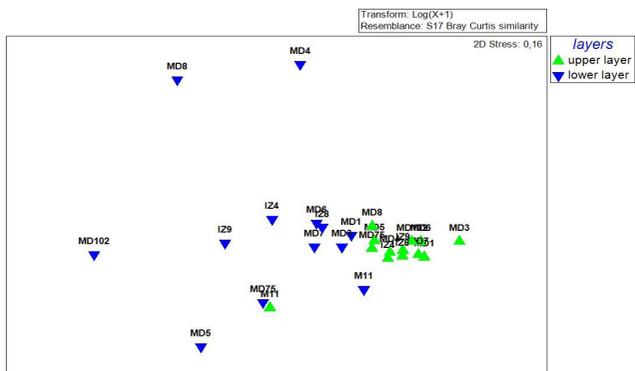
**Figure 5.** MDS analyses by seasons (1: February. 2: May. 3: July. 4: December)



**Figure 6.** Seasonal distribution of zooplankton abundance (ind.m<sup>-3</sup>) in the upper and lower layers at various sampling stations.



**Figure 7.** The variation in the number of species in the lower and upper layers at the sampling stations.



**Figure 8.** MDS ordination of combined data of upper- and lower layer samples.

In the Eastern Marmara Sea, zooplankton are more abundant in the upper layer than in the lower, which agrees with previous studies (Tarkan et al., 2005; İsinibilir et al., 2008, 2011). Strong stratification in the basin, depending on temperature and salinity, limits the daily vertical migration pattern of zooplankton (İsinibilir et al., 2011). Accordingly, species compositions differ between layers, and the main zooplankton biomass is concentrated in the upper layer (Mutlu, 2005; İsinibilir Okyar, et al., 2015). Thus, this study further corroborates that the top layer has larger values in terms of biomass and abundance than the lower layer. *Oithona* is a widespread genus in eutrophic and degraded unstable environments due to its resilience (Richard & Jamet, 2001; Castellani et al., 2005; İsinibilir et al., 2016; Svetlichny et al., 2018). In particular, *O. davisae*

and *O. nana* are classified as polluted area species (Drira et al., 2017; İsinibilir et al., 2008, 2016). *O. davisae*, a cyclopoid copepod originating from the western Pacific Ocean (Hirakawa, 1988), was first recorded in the Black Sea in the Sevastopol Bay in December 2001 (Zagorodnyaya 2002). Over the last decades, this species gradually spread to the entire eastern and western coasts of the Black Sea (Altukhov et al., 2014; Gubanova & Altukhov, 2007; Mihneva & Stefanova, 2013), the Sea of Marmara (Doğan & İsinibilir, 2016; İsinibilir et al., 2016) and the Aegean Sea (Terbiyik-Kurt & Beşiktepe, 2019). Due to its thermophilic nature (Svetlichny et al., 2016), this species is most abundant during the warm seasons and least abundant during winter and early spring (Altukhov et al., 2014; Mihneva & Stefanova, 2013; Uye & Sano, 1998; Zagami et al., 2018). However, variations in the population density of *O. davisae* showed differences across regions (Ambler et al., 1985; Uye and Sano, 1995; Svetlichny et al., 2018). In this study, *O. davisae* was distributed in eutrophic coastal areas and less abundant only in December. As *O. nana* is a tolerant, opportunistic, and widely adapted species, this species can be found in sea ports and near urban wastes and/or brackish waters with varying degrees of pollution (Richard and Jamet, 2001; Beşiktepe et al., 2023). As in previous studies on the Sea of Marmara (İsinibilir et al., 2008, 2016; İsinibilir Okyar et al., 2015), this work showed that *O. nana* is the predominant zooplankton species year-round, especially in severely perturbed coastal areas.

*A. clausi*, widely distributed in temperate waters and dominates zooplankton in polluted areas, is also the most prevalent species in the Sea of Marmara year-round (Gubanova et al., 2001; İsinibilir et al., 2008; 2011; Svetlichny et al., 2022, Beşiktepe et al., 2023). This study supports previous findings that *A. clausi* predominates in coastal areas, reflecting the eutrophic characteristics of this sea. On the other hand, *O. similis* and *P. parvus*, the other dominant copepod species in the Marmara Sea (İsinibilir et al., 2008; İsinibilir, 2009; 2010), are nonsensitive species that can live in both polluted and unpolluted marine environments (Drira et al., 2017).

Environmental factors such as temperature, salinity, predation, food availability, and water transparency can affect the Cladocera population (Calbet et al., 2001; Marques et al., 2006; Atienza et al., 2008; İsinibilir et al., 2011). At different spatiotemporal scales, the dynamics of small-sized cladoceran abundance are influenced by predatory forces from planktivorous fish and invertebrates (Onbe & Ikeda 1995, Egloff et al., 1997; Camatti et al, 2008). In this study, the increase in *P. avirostris* abundance was triggered by water temperature, whereas salinity caused the reverse effect. In addition to being an important food source for marine pelagic fish, *P. avirostris* plays an important role in the zooplankton community due to its high abundance in tropical and temperate waters, especially during the summer (Calbet et al., 2001; Marazzo & Valentin, 2003; Rose et al., 2004). As the body length of *P. avirostris* varies between 0.70 and 1.09 mm (Zhou et al., 2022), this species may be important for fisheries by attracting many larvae and adult pelagic fish such as mackerel, sardine, horse mackerel, and anchovy (Wu et al., 2023). *Penilia avirostris* exhibits a broad dietary spectrum, encompassing small diatoms and bacterivorous microflagellates (Turner et al., 1988) as well as prymnesiophyceans (Paffenhofer & Orcutt, 1986) and

bacteria (Lipej et al., 1997). The contributions of predation to the formation of marine zooplankton ecosystems have previously been disregarded (Verity & Smetacek, 1996). However, fish larvae, chaetognaths, and ctenophores are among the predators that might wipe out marine cladoceran populations (Duró & Saiz, 2000; Barz & Hirche, 2005). Nevertheless, how predation affects *P. avirostris* population dynamics in the Sea of Marmara is unclear. Other important cladoceran species like *Pleopis polyphemoides*, the most euryhaline species (Viñas et al., 2007), transpired throughout the winter months after going dormant during the warmer ones, as in previous studies (Isinibilir et al., 2008; Isinibilir, 2009; Isinibilir Okyar et al., 2015).

In the Sea of Marmara, *N. scintillans* exhibits a year-round distribution, with the greatest abundance in the spring and a secondary increase in the autumn (November) (Isinibilir et al., 2008; Isinibilir 2009). This species competes with zooplankton for food, and its high tolerance for temperature and salinity, coupled with its feeding on zooplankton eggs, promote its ecological success (Schaumann et al., 1988; Kirchner et al., 1996; Elbrächter & Qi, 1998; Quevedo et al., 1999). Thus, it is crucial to monitor the distribution and abundance of this species in the Sea of Marmara.

## CONCLUSION

In conclusion, the zooplankton communities in the Marmara Sea exhibited notable seasonal fluctuations in abundance and species diversity. Given the correlations between seasonal changes in dominant species and environmental factors such as temperature, salinity, and chlorophyll-a, zooplankton can serve as a significant indicator of changes in this marine environment. The Sea of Marmara has undergone significant changes recently due to rising temperatures and increased industrial pollution, which negatively influence zooplankton ecosystems. Extensive research conducted at the basin level is still required to elucidate the evolution and changes of this ecosystem that is vulnerable to human impacts and climate change.

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**Ethics Committee Approval:** The authors affirm that ethical approval is unnecessary for this study.

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## Post-Mucilage Distribution, Daily Growth, Mortality, and Hatch Date Timing of Sand steenbras *Lithognathus mormyrus* (Linnaeus, 1758) Juveniles in the Sea of Marmara

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### ABSTRACT

Between 2021-2022, a dense mucilage disaster occurred in the Sea of Marmara in Türkiye. Mucilage mostly covered coastal areas, which juvenile fish use for nurseries, growth, and protection. Due to Sand steenbras having been suggested as an environmental bioindicator species for Mediterranean coastal waters, this study attempts to reveal post-mucilage distribution and potential differences on the timing of hatching, daily age, growth, and mortality as revealed by the otolith microstructure. Individuals were sampled using beach seine nets from 12 equally spaced stations along the Sea of Marmara. Its presence and significant abundance in 10 out of 12 stations showed the Sand steenbras to be a common juvenile species for the Sea of Marmara. Hatching occurs between May-January, with peak hatching occurring in October. When considering that the mucilage had completely disappeared from the environment in August, the first set of hatching months can be seen to coincide with the dense mucilage. Thus, Sand steenbras can be considered as able to reproduce under these environmental conditions. The daily age of the Sand steenbras juveniles ranged between 38-235 days, with a mean of  $120.3 \pm 1.8$  days. The daily growth and mortality rates were also calculated respectively as 0.226 mm/day and 4.11%.

**Keywords:** Hatch date distribution, daily growth, juvenile fish, spatial distribution, mucilage

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### INTRODUCTION

The Sea of Marmara is a semi-enclosed basin located between the Black Sea and the Aegean Sea that connects them through the respective Istanbul and Dardanelles Straits. The variable physicochemical conditions of Marmara Sea such as global warming-related changes (Savun-Hekimoğlu & Gazioğlu, 2021), fluxiation (Altıok & Kayışoğlu, 2015), and pollution (Bilgili et al., 2022) cause undesirable environmental problems such as mucilage. Mucilage is defined as massive gelatinous aggregates that are produced by marine organisms under these afore-

mentioned undesirable conditions (Mecozzi et al., 2001). A dense mucilage event occurred in the Sea of Marmara between April-July, 2021. In the first months, the mucilage covered relatively all surface areas before becoming a submersion. The coastal areas later were completely covered by mucilage in May and June.

Mucilage has direct and indirect effects on aquatic living organisms. Mucilage directly affects sessile animals such as corals, sponges, mussels, and anemones by covering them. These animals are suspension-feeders, and water flow is crucial to their survival. In addition,



marine algae and plants need light for photosynthesis. Thus, the adverse effects of mucilage on these organisms are fatal and inevitable. The indirect effects of mucilage may occur on other animals such as gastropods, decapods, cephalopods, and fish. However, relatively few studies have been conducted on this. Ertürk-Gürkan et al. (2022) found the mucilage to be consumed as a food source by benthic species *Eriphia verrucosa*. Dalyan et al. (2021) studied the effects of mucilage on cryptobenthic adult fish assemblages in the North Aegean Sea where mucilage was less impactful and stated these species to have had to change their habitats due to mucilage. Even worse, Karadurmuş and Sarı (2022) revealed mass deaths for 12 teleost, two cartilaginous, and four decapod species in the Sea of Marmara.

Due to mucilage occurring mostly along coastal areas which are known as nursery areas for new settlers and juveniles, understanding the mucilage-juvenile fish relationship may be important. Because fish in their early life stages have only been alive for a short time, retrospectively monitoring the effects of sudden environmental conditions may be more logical as these carry more noticeable signs regarding adult fish. Micro-increment studies of the sagittal otolith allows one to determine the daily age and the related time when hatched. Some pollution- and environmental change-related variations on these parameters have been previously examined (Campana, 1984; Meier et al., 2010; Isnard et al., 2015; Sardi et al., 2021) for some fish larvae and juveniles. Due to most teleost fish laying their eggs on surface areas and the fertilization and development of eggs occurring in a pelagic environment, the success of these development process is questionable in the environments where mucilage occurs. This study hypothesizes that eggs may not be fertilized as a result of the dense mucilage aggregates and the need for external fertilization. In addition, hatching larvae may get stuck in the mucilage and fail to move. Larvae may also not be able to find prey after their mouth gap opens. All these possibilities may result in high mortalities. To understand these effects, the species should be selected as a reference as their spawning season overlapped with the occurrence of dense mucilage. Thus, the study has selected Sand steenbras both for having been suggested as an environmental bioindicator species for Mediterranean coastal waters (Tom et al., 2003; Funkenstein et al., 2004) as well as for the overlap their reproduction cycle had with the dense mucilage event.

Thus, this study aims to reveal evidence for the spawning that has occurred using the spatial distribution of biomass and hatch date distributions. In addition, the study attempts to observe potential differences regarding the daily age, growth, and mortality rates compared to previous studies.

## MATERIALS AND METHODS

*L. mormyrus* juveniles were collected using beach seine sampling from 12 equally spaced stations located along the coast of the Sea of Marmara (Figure 1). Monthly beach seine sampling hauls were carried out with two replications from each station between December 2021-March 2022. The length of wings, the height of the wings, and the length, width, and height of the bag of the beach seine were rigged as 30 m, 1.8 m and 2x2x2 m, respectively. The 4 mm nominal bar length was used in the bag net,

whereas a 6.5 mm nominal bar length was used in the wing net (Figure 2). The beach seine hauls were conducted on seagrass beds and sandy habitats. Specimens were kept on ice packs and transported immediately to the laboratory. The individuals were then measured to the nearest 0.1 cm regarding total length (TL) and weighed to the nearest 0.01 g regarding total weight (W). The mean abundance at each location was given with the catch per unit effort (CPUE) based on the number of fish per haul. The CPUE was calculated with the following equation:

$$CPUE (n / haul) = N_i / H_i \quad (1)$$

where  $N_i$  is the total individual number of Sand steenbras obtained from station  $i$  in the study period, and  $H_i$  is the total number of hauls from station  $i$ . The total number of hauls was calculated by multiplying the replication number (equal to 2) with the total monthly survey number (equal to 4).

The length-weight relationship parameters are calculated using Le Cren's (1951) formula:

$$W = a * TL^b \quad (2)$$

where  $W$  is the total weight (g),  $TL$  is the total length (cm), and  $a$  and  $b$  are regression parameters. The growth type was identified using Student's t-test in accordance with the following equation (Sokal & Rohlf, 1987):

$$ts = (b - 3) / SE_b \quad (3)$$

where  $ts$  is the t-test value,  $b$  is the slope, and  $SE_b$  is the standard error of the slope. Student's t-test examines the significance of the difference of  $b - 3$ , which represents isometric growth (Pauly, 1984).

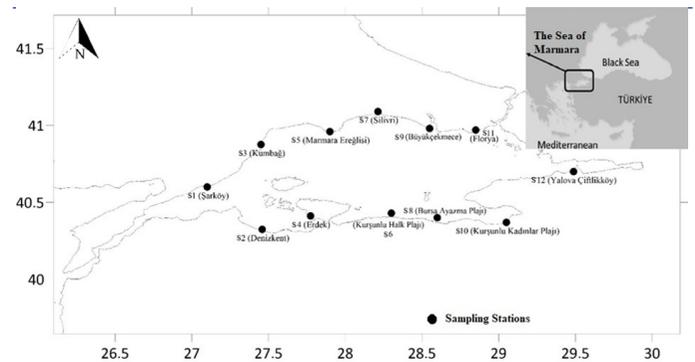


Figure 1. Study area and sampling stations.

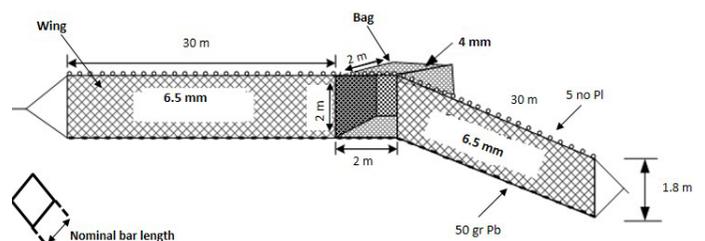


Figure 2. Technical plan of beach seine net.

A linear regression analysis ( $y = bx + a$ ) was used to determine the relationships between  $TL$  (total length) and  $FL$  (fork length), as well as  $TL$ -otolith length ( $OL$ ), total weight ( $TW$ )-otolith weight ( $OW$ ),  $TL$ -otolith width ( $OW_i$ ),  $TL$ - $OW$ ,  $OL$ - $OW_i$ , and  $OL$ - $OW$ .

For the otolith microstructure analyses, the sagittal otoliths of *L. mormyrus* juveniles were grounded and polished with abrasive papers. The sagittal otoliths of *L. mormyrus* were removed using forceps. The right otoliths were placed on a glass slide with thermoplastic glue for viewing under a microscope. After grounding and polishing, increment rings were counted from the first visible check mark succeeding the primordium to the outer edge along the maximum diameter axis, as described by Brothers (1984; Figure 3).

The daily growth rate ( $GR$ ) of *L. mormyrus* was calculated based on simple linear regressions using the least-squares method between the larval lengths ( $SL$ ) and juvenile lengths ( $TL$ ) with respect to age in days (Leonarduzzi et al., 2010) according to the following formula:

$$L = a + b(t) \quad (4)$$

where  $L$  is the larval and juvenile lengths,  $a$  is a constant,  $t$  is a function of age in days, and  $b$  is the daily growth rate (mm). The daily mortality rates were estimated using the slope coefficient in the regression relationship of the natural log values of the abundance-per-length groups (Pauly, 1984). Hatching time was determined by subtracting the age in days of the individual from the sampling date, and the hatch peak and hatch interval were determined in order to apply the calculation over all sampling months. Dissolved oxygen, salinity, and temperature were measured using a YSI 6600 (6-series multiparameter water quality sondes).



**Figure 3.** A 36 mm total length juvenile Sand steenbras individual and sagittal otolith at 90 days old.

## RESULTS AND DISCUSSION

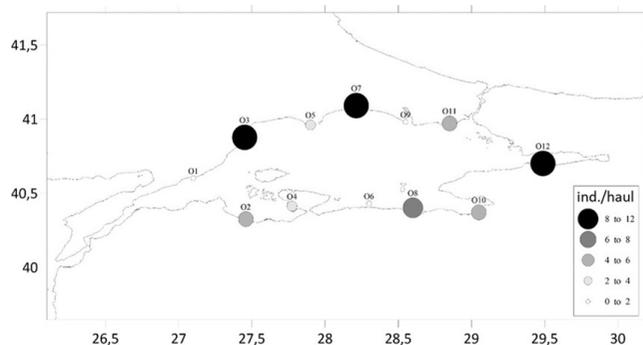
In terms of the spatial variation of abundance, *L. mormyrus* juveniles were detected in 10 of 12 stations in the Marmara Sea. *L. mormyrus* juveniles were not found in Stations S9 (Büyükçekmece) or S6 (Bandırma Kurşunlu Shores). Stations S3 (Kumbağ) and S12 (Yalova Tıgем) had the highest abundance with a mean of 11.6 ind./haul. Station S7 (Silivri) also had a high abundance (9.1 ind./haul). As can be seen in Figure 4, *L. mormyrus* juveniles were well distributed throughout all of the Marmara Sea.

Table 1 summarizes the minimum (min.), maximum (max.) and mean ( $M$ ) lengths for  $TL$  and  $FL$ , as well as the weight values of the 479 individuals. The highest individual number, mean  $TL$ , and mean  $FL$  were detected in January, whereas the max. weight was found in February. The length-frequency distribution showed 27% of the individuals to be found in the 53-57 mm  $TL$  group. The lowest size (28-32 mm) and highest size (73-77 mm)  $TL$  groups were represented with low individual numbers due to selectivity and recruitment patterns.

The length frequency distribution shows two different peaks, accordingly two cohorts in one spawning season (Figure 5). The length-weight relationship curve can be seen in Figure 6. The  $b$  value was found to significant vary from 3 ( $t = 2.41$ ;  $t$ -table = 1.96;  $t > t$ -table;  $p < 0.05$ ) and to exhibit negative allometric growth (Table 2).

The measurements related to sagittal otolith length ( $OL$ ), otolith width ( $OW_i$ ), and otolith weight ( $OW$ ) of the *L. mormyrus* juveniles are shown in Table 3. A linear relationship has been detected between  $TL$ - $FL$ ,  $TL$ - $OL$ ,  $TW$ - $OW$ ,  $TL$ - $OW_i$ ,  $TL$ - $OW$ ,  $OL$ - $OW_i$ , and  $OL$ - $OW$ . The equations and related parameters are summarized in Table 4.

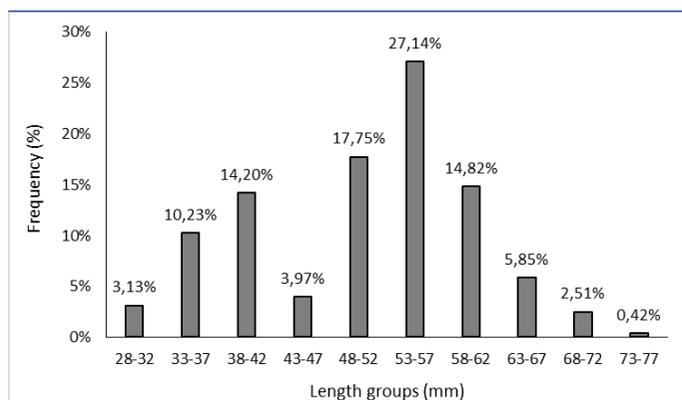
The pattern for age in days as estimated by the sagittal otoliths of *L. mormyrus* juveniles ranged between 38-235 days. The mean age in days of *L. mormyrus* was determined as  $120.3 \pm 1.8$ . The youngest individual was sampled on December 16, 2021, whereas the oldest one was sampled on February 18, 2022. The 120-139 (22.8%) age-in-days grouping was determined to be the dominant age group, followed by the 140-159 (17%) age-in-days grouping. The age in days-length key for juvenile *L. mormyrus* is shown in Table 5.



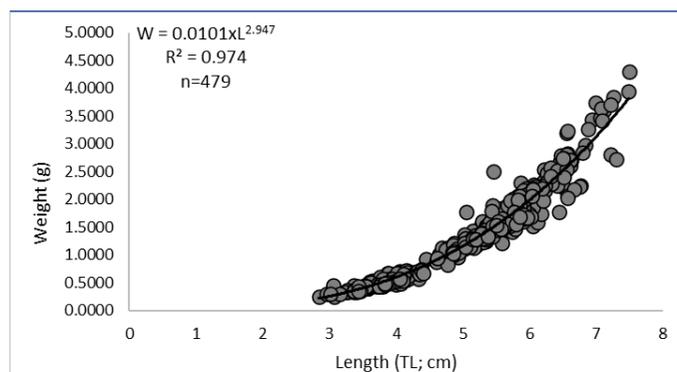
**Figure 4.** Spatial variation of mean CPUE of the *Lithognathus mormyrus* in the Sea of Marmara.

**Table 1.** The length and weight values and temporal variations of juvenile Sand steenbras (*Lithognathus mormyrus*).

		December	January	February	March	Total
Total Length (TL; mm)	Individual	146	149	127	57	479
	Min.	28.42	30.73	30.13	34.39	28.42
	Max.	72.61	74.88	74.84	65.04	74.88
	M	47.38	53.24	52.36	50.99	50.95
	SE	0.96	0.69	0.79	0.94	0.45
	SD	11.65	8.36	8.85	7.07	9.79
	CI	47.38 ± 1.58	53.24±1.13	52.36±1.30	50.99±1.58	50.95±0.74
Fork Length (FL; mm)	Individual	146	149	127	57	479
	Min.	26.45	28.52	28.1	32.25	26.45
	Max.	69.09	71.01	68.88	59.95	71.01
	M	43.81	50.02	48.7	46.98	47.46
	SE	0.95	0.64	0.73	0.89	0.43
	SD	11.49	7.77	8.22	6.76	9.42
	CI	43.81±1.56	50.02±1.05	48.7±1.20	46.98±1.50	47.46±0.71
Weight (g)	Individual	146	149	127	57	479
	Min.	0.2439	0.2445	0.2967	0.3479	0.2439
	Max.	3.8538	4.2984	3.9433	2.4182	4.2984
	M	1.23	1.46	1.47	1.37	1.37
	SE	0.075	0.049	0.063	0.064	0.033
	SD	0.91	0.6	0.71	0.48	0.73
	CI	1.23±0.12	1.46±0.08	1.47±0.10	1.37±0.11	1.37±0.05



**Figure 5.** Length-frequency distribution of juvenile Sand steenbras (*Lithognathus mormyrus*).



**Figure 6.** Length-weight relation of juvenile Sand steenbras (*Lithognathus mormyrus*).

**Table 2.** The length-weight relationship parameters of Sand steenbras *Lithognathus mormyrus* juveniles.

a*	b*	SE(b)*	R <sup>2</sup> *	Growth Type	SD <sub>a</sub>	SD <sub>b</sub>
0.0101	2.947	0.02194	0.98	Negative allometric	0.00945 - 0.01087	2.9043 – 2.9906

\*a and b are regression parameters; SE = standard error of the slope (95% CI); R<sup>2</sup> = coefficient of determination; SD<sub>a</sub> = standard deviation of a (95% CI); SD<sub>b</sub> = standard deviation of b (95% CI).

The daily growth rate of the species was found as 0.226 mm/day according to the linear regression between the age in days and juvenile lengths (Figure 7). The instantaneous and mean daily mortality rates of juvenile *L. mormyrus* were calculated as 0.0403 and 4.11%, respectively (Figure 8).

By subtracting the age in days from the time of sampling, the hatching of *L. mormyrus* was determined to have occurred between May 2021-January 2022, with an increase occurring between August-October and peaking in October (Figure 9).

**Table 3.** Otolith length (OL), otolith width (OWi), and otolith weight (OW) of Sand steenbras (*Lithognathus mormyrus*) juveniles.

	Individual	Min.	Max.	Mean	SE	SD
Otolith Length (OL, mm)	200	1.282	3.387	2.331	0.032	2.331 ± 0.053
Otolith Width (OWi, mm)	200	0.885	1.982	1.475	0.018	1.475 ± 0.030
Otolith Weight (OW, g)	200	0.0001	0.0042	0.0024	0.0001	0.0024 ± 0.0001

\*OL = otolith length; OW = otolith weight; OWi = otolith width; SE = standard error; SD = standard deviation of b (95% CI).

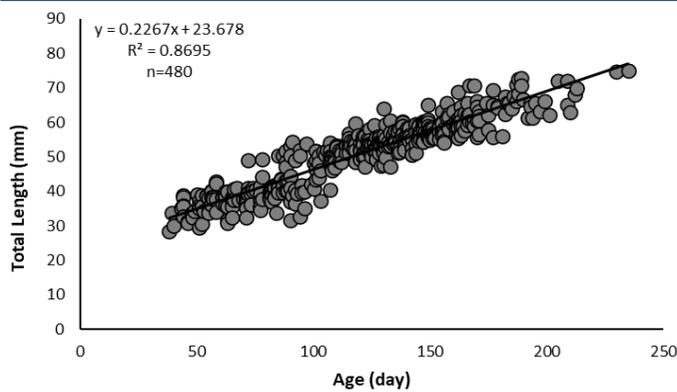
**Table 4.** Linear Regression parameters of body measurements and sagittal otoliths of Sand steenbras (*Lithognathus mormyrus*) juveniles.

Relationship	Equation	SE <sub>b</sub> *	R <sup>2</sup> *	95% SD <sub>b</sub> *	
TL*-FL*	FL = 0.9468TL - 0.9367	0.0073	0.99	0.9322	0.9610
TL-OL*	OL = 0.0398TL + 0.3117	0.154	0.88	0.0377	0.0419
TW*-OW*	OW = 0.0011TW + 0.0008	0.0006	0.66	0.00101	0.00123
TL-OWi*	OWi = 0.0219TL + 0.3687	0.091	0.87	0.0207	0.023
TL-OW	OW = 9E-05TL - 0.0023	0.0004	0.83	8.63E-05	9.82E-05
OL-OWi	OWi = 0.5376OL + 0.2366	0.073	0.92	0.5133	0.558
OL-OW	OW = 0.0022OL - 0.0029	0.0004	0.86	0.002112	0.002365

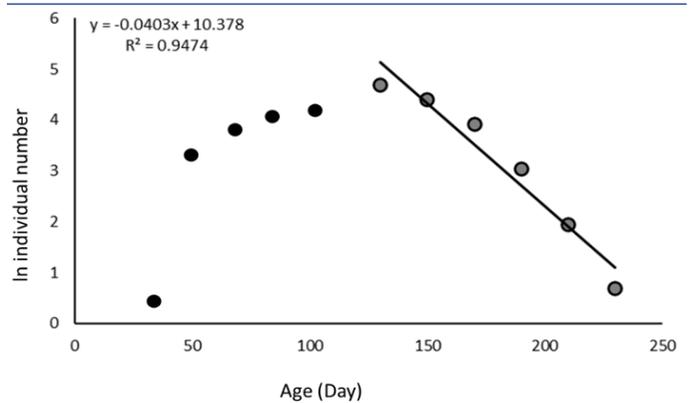
\*TL = total length; TW = total weight; FL = fork length; OL = otolith length; OW = otolith weight; OWi = otolith width; SE<sub>b</sub> = standard error of the slope (95% CI); R<sup>2</sup> = coefficient of determination; SD<sub>b</sub> = standard deviation of b (95% CI).

**Table 5.** The age-length key of juvenile *Lithognathus mormyrus* in the Marmara Sea, Türkiye.

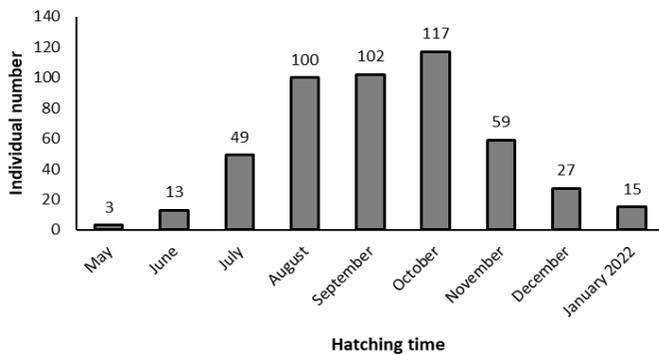
Length (mm)	Age in days										
	20-39	40-59	60-79	80-99	100-119	120-139	140-159	160-179	180-199	200-219	220-239
25-29	1	1									
30-34	1	14	5	4							
35-39		21	33	19	1						
40-44		4	9	23	4						
45-49			2	3	19	8					
50-54				9	31	73	12				
55-59					4	24	61	21	1		
60-64						4	7	21	6	2	
65-69							2	6	10	3	
70-74								2	4	2	2



**Figure 7.** Age-length relationship of juvenile Sand steenbras (*Lithognathus mormyrus*).



**Figure 8.** Linearized catch curve of juvenile Sand steenbras (*Lithognathus mormyrus*).



**Figure 9.** Hatching time of juvenile Sand steenbras (*Lithognathus mormyrus*) in the Marmara Sea.

The detection of juvenile individuals for each age-in-days class with a significance abundance from each location in the present study may be an indication that spawning might have occurred in the area and the individuals had not been sampled accidentally. According to the microstructure of the sagittal otoliths, the time of hatching had occurred between May-January, with peak hatching having occurred in October. When considered that the mucilage had completely disappeared from the environment in August, the first hatching months can be seen to have coincided with the dense mucilage. Thus, Sand steenbras can be considered as able to reproduce under these environmental conditions. Thus, the hypothesis related to "spawning being prevented due to unsuccessful external fertilization and being stuck in mucilage" is rejected.

Recruitment lengths of Sand steenbras have been stated as 60-70 mm TL (Matic-Skoko et al., 2007) and 72 mm TL (Lasiak, 1986). Ayyıldız et al. (2014) also only found 14 of 416 individual specimens to be longer than 75 mm TL. Ayyıldız and Altın (2021) additionally measured the largest juvenile individual they found as 74 mm TL. The largest individual found in the present study measured was 75 mm TL, which coincides with the findings of previous studies and confirms the relative recruitment length of *L. mormyrus*. Also, the well-distributed age and size class under 75 mm TL in the present study may be an indication that recruitment is able to occur. The spawning frequency of adult *L. mormyrus* identified has been identified as one clear seasonal peak per year (Bauchot & Hureau, 1986). Hereby, age 0 cohorts with different ages in days within a certain period of this single reproductive period need to be observed as juvenile individuals. The hatch date distribution and age-length data set presented in this study have revealed a single cohort. This may be proof of the spawning success of adult *L. mormyrus* under mucilage conditions.

Some population parameters related to stock status, such as growth type, condition factors, and daily growth and mortality rates were estimated and compared with previous studies. Matic-Skoko et al. (2007) examined the growth of juvenile *L. mormyrus* ranging from 8 mm-103 mm in length around the Adriatic Sea. They found the  $b$  exponent estimated from the length-weight relationship to be 3.141 and calculated the condition factor ( $CF$ ) as 1.245. Ayyıldız and Altın (2021) calculated the  $b$  value as 3.106 in the Gökçeada Island of the North Aegean Sea. Reis and Ateş (2020) also found the  $b$  value for immature individuals to be 3.276

in the Köyceğiz Lagoon. Meanwhile, the current study detected  $b$  and  $CF$  as 2.947 and 0.94 ( $SD \pm 0.04$ ), respectively. The higher  $b$  and  $CF$  values in the Adriatic Sea, North Aegean Sea, and South Aegean Sea indicate those stocks to perhaps have better conditions compared to the Marmara Sea. Ayyıldız et al. (2014) evaluated the growth parameters of juvenile *L. mormyrus* around the Dardanelles Strait in Türkiye. The distribution for age in days was seen to closely correspond to length. Conversely, they found a higher mean daily growth rate (0.325 mm/d) compared to the current study's finding (0.226 mm/d). In addition, they found relatively lower daily mortality rates (2.16%) compared to the current study's result (4.11%). Ayyıldız and Altın (2021) also detected a higher mean daily growth rate (0.317 mm<sup>-1</sup>) and relatively comparable mortality rate (4.61%) around Gökçeada Island of the North Aegean Sea. Some negative variations related to stock health such as conditions, growth and mortality were also detected. These differences may be a result of the species' feeding style, which feed by scratching the seabed. Reaching food may have been difficult due to the bottom being covered by mucilage. Also, the differences may be related to many other variables such as variations regarding the physicochemical parameters stemming from temporal and geographical differences, food availability, and pollutants along coastal areas. Thus, making a definitive judgement is difficult due to the studies not have been conducted simultaneously.

The timing of hatchings first occurred in May and peaked in October in the present study. The peak hatching period had been determined as June-July in the Adriatic Sea (Matic-Skoko et al., 2007), as August in the Dardanelles Strait (Ayyıldız et al., 2014), and between July-December in the North Aegean Sea (Ayyıldız & Altın, 2021). The main parameter controlling the spawning activity of Sand steenbras has been stated as sea surface temperature (SST; Vitale et al., 2011). Mandić et al. (2014) studied an ichthyoplankton community in Boka Kotorska Bay (South Adriatic Sea) and stated Sand steenbras to dominate the ichthyoplankton biomass in April, when their measured SST values ranged between 14.6°C-16.4°C ( $M = 15.5^\circ\text{C}$ ). According to Karadurmuş and Sarı (2022), the SST ranged between 14.5°C and 15.6°C in the Marmara Sea during the dense mucilage on April 25, 2021. The estimated onset of hatching occurred in May 2021. When additionally examining the fertilization period, the onset of hatching in May overlaps exactly with the species SST preferences for spawning. The onset of hatching, which seems to have been delayed, may actually have been caused not by the mucilage but by the factors that had caused the mucilage. In addition, hypoxia was reported (dissolved oxygen [DO] ranging between 2.3 mg/l-3.6 mg/l) in the Marmara Sea in April 2021 (Karadurmuş & Sarı, 2022), just before the hatching. Meanwhile, this study measured DO as ranging between 6.5 mg/l-9.4 mg/l ( $M = 8.4$  mg/l) between December 2021-March 2022. As the mucilage density decreased, the oxygen values were observed to have increased. Thus, another possibility for the slightly delayed hatching and/or spawning times may have been caused by low oxygen levels.

## CONCLUSION

Whether the differences mentioned in this study are directly related to the mucilage effect or the environmental conditions that

caused the mucilage to appear remains unclear. To better understand the direct and indirect effects of environmental factors such as mucilage on fish and their early life stages, factors such as distribution, feeding, reproduction, behavior, and pollution should be monitored more closely during and immediately after a mucilage event. Using back-calculated estimations based on the micro-increments of juvenile otoliths becomes more accurate when considering the obtained data alongside observations that were made during an incident. Simultaneously taking samples from areas affected by mucilage and unaffected areas should also increase the accuracy of back-calculation estimations. The world may encounter similar environmental disasters more frequently due to pollution and global warming, which are expected to increase in the near future. More detailed studies should be performed for understanding the direct and indirect effects environmental disasters such as mucilage events have on the early life stages of teleost fishes.

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# Reproductive Biology of the Catfish *Pachypterus atherinoides* (Bloch, 1794) with Special Reference to Its Lentic and Lotic Ecosystems

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## ABSTRACT

The study of reproductive biology is extremely crucial in fishery. *Pachypterus atherinoides* (Bloch, 1794) is a good food as well as an ornamental catfish in the World. The present study aims to examine the seasonal reproductive behavior of *P. atherinoides* regarding both its lentic and lotic ecosystems. The study was conducted over two years in selected lentic and lotic aquatic ecosystems of the Jhargram and Paschim Medinipur districts of West Bengal, India using the seasonally observed gonadosomatic index (GSI), fecundity, and gonad maturation stages for both habitats in the study area. The study reveals *P. atherinoides* to show single annual spawning between June-September. No significant difference was found regarding breeding period in terms of lentic and lotic aquatic ecosystems. This study can help aquaculturists regarding the culture, captive breeding, and conservation of *P. atherinoides*, as well as researchers regarding the biometric study of another fish.

**Keywords:** Fecundity, gonadosomatic index, gonad maturation, lentic, lotic, *Pachypterus atherinoides*

## INTRODUCTION

Fish reproductive biology entails gonad maturation, spawning period, spawning frequency, fecundity, and sexual development. Breeding biology, maturation, and fecundity are investigated in order to provide data that can be useful regarding fish conservation. The frequency of spawning in a year for a specific fish species can be determined by knowing its spawning periodicity. The significance of fecundity is self-evident, as it indicates the number of eggs that are laid. Understanding the fecundity of fish facilitates setting the arrangements for effectively hatching eggs on a fish farm. This type of research is also critical in fishery management. Fundamental themes in fish biology also include the description of reproductive techniques and the study of fecundity. For one to understand a fish's life history, spawning, culture, and administration, one also needs to understand fish reproduction. Re-

productive strategies are one of the most important aspects of stabilizing a population in an environment. Examining the stages of gonad development for each fish, such as the period of the spawning season and the size at first maturity, is necessary for studying fish reproduction. The study of reproductive behavior has focused primarily on fish fecundity and the gonadosomatic index (GSI). The GSI is a key component of fish biology and aids in identifying the fish mating season by offering a full grasp of fish reproduction and the reproductive condition of each species (Shankar & Kulkarni, 2005). Fecundity is the quantity of maturing eggs a female has before spawning (Bagenal, 1978). Having a comprehensive understanding of fecundity is important for estimating the commercial potential of fish stocks and the spawning stock's abundance and reproductive potential (Lagler et al., 1956). *Pachypterus atherinoides* (Bloch, 1794), often known as Indian potasi, is a common cat-

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fish belonging to the family Schilbeidae and is found in rivers and ponds throughout India (except Kerala), Pakistan, Bangladesh, Nepal, Myanmar, and Burma (Menon, 1999). It has high nutritional as well as ornamental value. The fish meal of the species contains 343 Kcal in energy, 16 g of water, 58 g of protein, 9 g of fat, and 17 g of minerals (primarily 1,597 mg of calcium, 595 mg of phosphorus, and 41 mg of iron) per 100 g (Gopalan et al., 2004). Aquarists are attracted to it because of its bright color and small size. This species has been gradually decreasing due to pollution, habitat destruction, selective captive breeding, and removal from aquatic bodies due to their carnivorous nature, as well as the high demand for exotic catfish (African catfish *Clarius gariepinus* [Burchell, 1822]; Thai catfish *Pangasius sutchi* [Fowler, 1937]). The world has seen very little research on the reproductive biology of *Pachypterus atherinoides* (Bloch, 1794). India has yet to have a study on the reproductive biology of *Pachypterus atherinoides*. Gogai et al. (2020) and Jana et al. (2024) only studied this species' feeding biology. Gosavi et al. (2020) studied the reproductive biology of *Pachypterus khavalchor* in the Panchaganga River of Western Ghat, India. Various researchers (Dasgupta, 2004; Chattopadhyay et al., 2014; Paul and Chanda, 2017; Gupta, 2015; Jana et al., 2021A; Jana et al., 2021B; Jana et al., 2022A; Jana et al. 2022B; Sit et al. 2020; Chanda and Jana., 2021; Sahil et al., 2023; Sit et al. 2022A; Sit et al. 2022B; Sit et al., 2023A; Sit et al., 2023B; Jana et al., 2024) have also studied some aspects of various indigenous fish species in West Bengal, but none of these observed the reproductive behavior of *Pachypterus atherinoides*. Therefore, the present study will depict the seasonal reproductive biology of two different habitats that will be important for the conservation and propagation of this species.

## MATERIALS AND METHODOLOGY

**Collection of fish specimens:** Specimens were collected every 15 days from the selected lentic (ponds) and lotic (river sites) habitats of the Paschim Medinipur and Jhargram districts of West Bengal, India, during the pre-monsoon (March-June), monsoon (July-Oct), and post-monsoon (Nov-Feb) seasons between March 2020-February 2022 (Figure 1).

**Sex determination:** Male and female specimens were identified based on genital papilla, with males having a distinct extended papilla (Figure 2).

**Length and Weight Measurements:** Seasonally and ecosystem/habitat-wise, each specimen's total weight and length were measured using a digital scale with 0.01 g accuracy and a digital slide caliper with 0.01 mm accuracy, respectively.

**Dissection and Internal Organ Measurement:** Gonads were collected by dissection with the help of scissors, forceps, needles, and a brush. Digital slide calipers were used to measure the length, and an electronic balance was used to weigh each gonad.

**Gonadosomatic Index (GSI):** Howaida et al.'s (1998) formula was used to determine the GSI as follows:

$$GSI = \frac{\text{Weight of gonad}}{\text{Weight of fish}} \times 100 \quad (1)$$

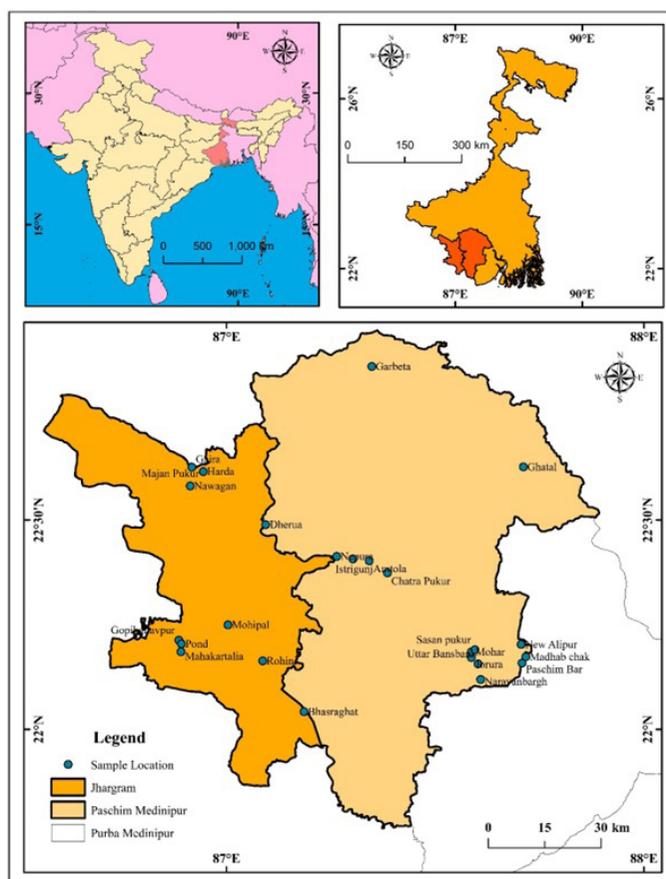


Figure 1. Specimen collection sites.

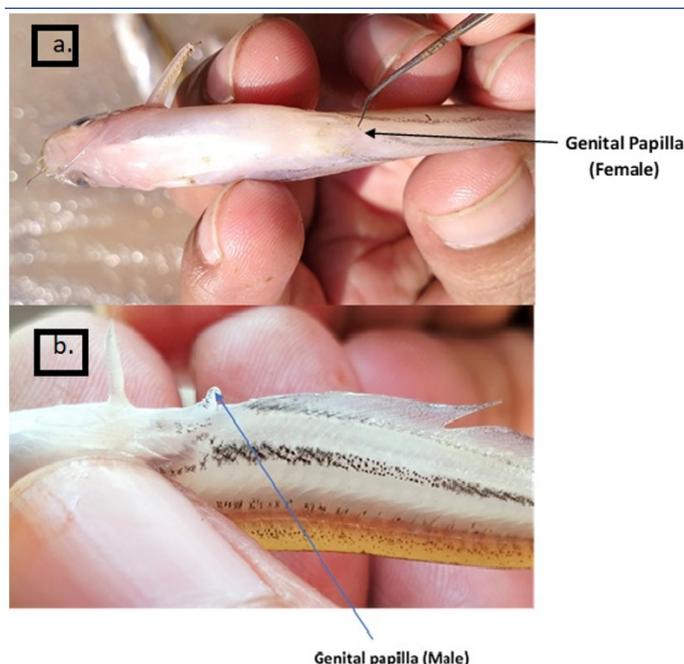


Figure 2. Genital apparatus of *P. atherinoides*. a.) Female; b.) Male.

**Fecundity:** The egg sac's front, middle, and posterior regions were divided into equal numbers of mature and immature eggs, which were then separated and counted using a needle and magnifying glass for each portion. The total number of eggs (i.e., the fish's fecundity) is measured using Dewan and Doha's (1979) gravimetric method as follows:

$$F = n G / g \quad (2)$$

where *F* is fecundity, *n* is the number of eggs in the subsample, *G* is the total weight of the ovary, and *g* is the weight of the subsample in grams.

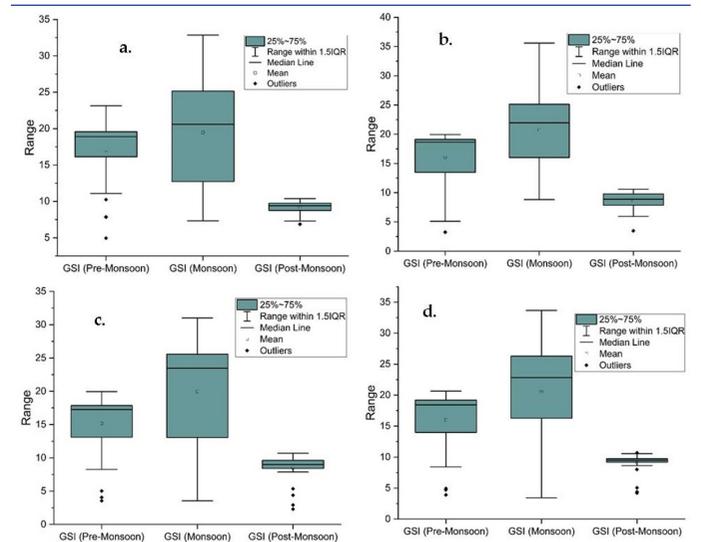
**Observed gonad maturation:** Examinations were carried out at the macro and microscopic (Model-XSP L101) levels to examine both species' cycles of gonad maturation. Female and male gonad maturation stages have been divided into the various stages of development (Heins & Baker, 1988; Gomes & Araaujo, 2004; Gupta & Banerjee, 2013).

**Data Analysis:** Lastly, the study analyzed the data using the Microsoft Excel (2019), SPSS (2021), and Origin Pro (2023) software systems.

**RESULTS AND DISCUSSION**

GSI values were highest during the monsoon season ( $GSI_{J-Lentic} = 20.29 \pm 6.926$  and  $GSI_{PM-Lentic} = 19.94 \pm 8.075$ ;  $GSI_{J-Lotic} = 20.72 \pm 6.29$  and  $GSI_{PM-Lotic} = 20.50 \pm 7.56$ ) followed by pre-monsoon ( $GSI_{J-Lentic} = 16.02 \pm 4.77$  and  $GSI_{PM-Lentic} = 15.166 \pm 4.52$ ;  $GSI_{J-Lotic} = 16.06 \pm 4.65$  and  $GSI_{PM-Lentic} = 15.99 \pm 4.91$ ), post-monsoon ( $GSI_{J-Lentic} = 8.85 \pm 1.24$  and  $GSI_{PM-Lentic} = 8.52 \pm 2.00$ ;  $GSI_{J-Lotic} = 8.65 \pm 1.46$  and  $GSI_{PM-Lotic} = 9.05 \pm 1.56$ ) in both habitats of the two districts (Table 1 & Figures 3-4). The maximum GSI in both habitats occurred from June to September, with the peak value occurring in July for the lentic habitats and August for the lotic habitats, while the lowest value was in December for both habitats (Figures 5-6). The GSI values indicate the breeding season to occur between June-September for *P. atherinoides*. Hossian et al. (2019) reported the GSI to be high between April-June and very low between

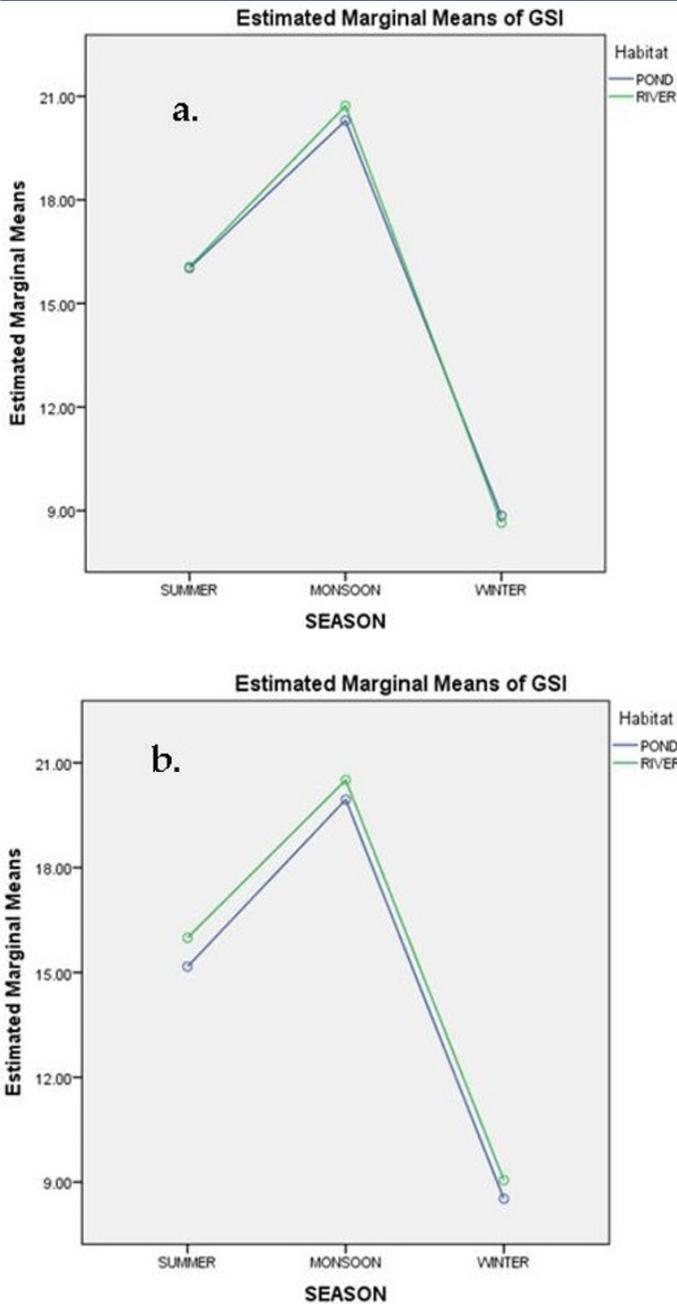
January-March, with the spawning season from April to June peaking in April and being at its lowest in May for the *P. atherinoides* from Kangsha River in Bangladesh. Paul et al. (2021) observed the same result as Hossian et al. (2019): After GSI peaked in April, it gradually decreased up to July. However, the present findings are not related to these two works due to the different physicochemical parameters. The current study measure fecundity as  $482 \pm 203.02$  (4.44g -6.57g TW) and  $492 \pm 236.91$  (4.31g -6.85g TW) for the respective lentic and lotic areas of Jhargram district; while this was measured as  $526 \pm 243.32$  (4.23g -6.77g TW) and  $538 \pm 245.12$  (4.36g -6.83g TW) for Paschim Medinipur's respective lentic and lotic areas (Table 2). Hossain et al. (2019) reported fecundity values of 1541-10,043 (2.33-5.59 g), while Paul et al. (2021) observed values of  $1,805.17 \pm 965.71$  to  $4,553.07 \pm 1,755.36$  for the *P. atherinoides* from Bangladesh. Gosavi et al. (2020) measured absolute fecundity to varied between 932-24,642 eggs/fish and relative fecundity to range between 226-



**Figure 3.** Seasonal GSI values of *P. atherinoides* in Jhargram (a & b) and Paschim Medinipur (c & d) districts: a & c are ponds; b & d are rivers.

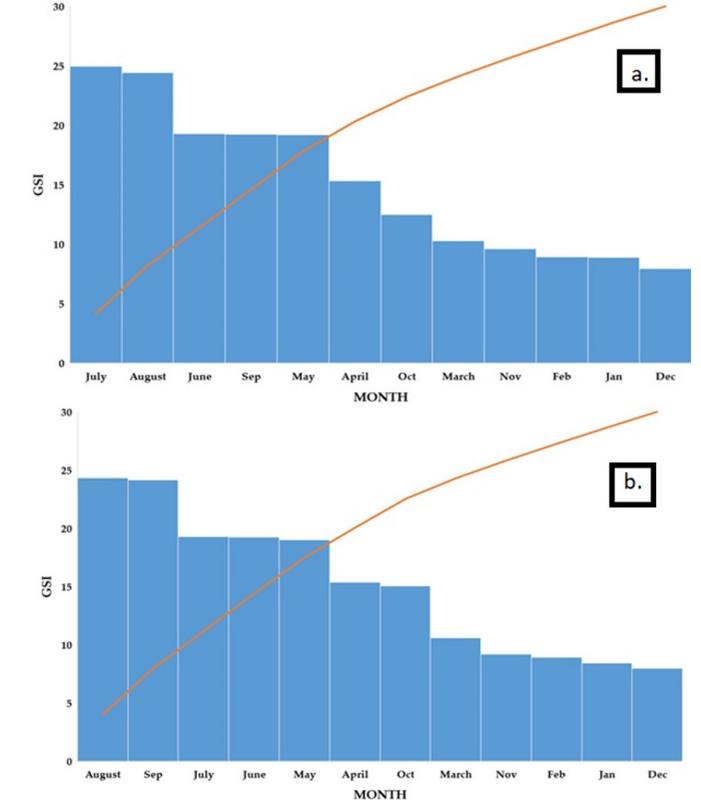
**Table 1.** Seasonal GSI of *P. atherinoides* for both lentic and lotic habitats in Jhargram and Paschim Medinipur districts (N = 128)

District	Habitat	Season	GSI			
			Min.	Max.	Mean	SD
JHARGRAM	Pond	Pre-monsoon	4.60	23.14	16.0244	4.77928
		Monsoon	7.33	32.87	20.2942	6.92698
		Post-monsoon	4.73	10.38	8.8555	1.24614
	River	Pre-monsoon	3.25	19.93	16.0655	4.65761
		Monsoon	8.82	35.60	20.7204	6.29151
		Post-monsoon	3.48	10.57	8.6504	1.46845
PASCHIM MEDINIPUR	Pond	Pre-monsoon	3.55	19.97	15.1664	4.52530
		Monsoon	3.55	31.02	19.9467	8.07572
		Post-monsoon	2.30	10.70	8.5228	2.00369
	River	Pre-monsoon	3.90	20.64	15.9921	4.91183
		Monsoon	3.44	33.67	20.5057	7.56303
		Post-monsoon	4.21	10.70	9.0554	1.56233



**Figure 4.** Seasonal variation of GSI for *P. atherinoides* in both habitats of Jhargram (a) and Paschim Medinipur (b) districts.

723 eggs/g of body weight for *Pachypterus khavalchor*. The study's measured fecundity values are lower than those from previous studies. The  $R^2$  values between gonad weight and fecundity were measured as 0.850 and 0.9047 for Jhargram's lentic and lotic areas and as 0.738 and 0.921 for Paschim Medinipur's lentic and lotic areas, respectively (Figure 8). The  $R^2$  values between body weight and fecundity were measured as 0.640 and 0.786 for Jhargram's lentic and lotic habitats and as 0.679 and 0.755 for Paschim Medinipur's lentic and lotic habitats, respectively (Figure 9). Fecundity was positively linearly correlated with gonad weight and body weight in both the studied habitats. The same



**Figure 5.** Monthly variation of GSI for *P. atherinoides* in the Jhargram district's: a.) ponds; b.) rivers.

result was observed by Hossain et al. (2019) and Paul et al. (2021) for the same species and by Islam and Das (2006), Siddique et al. (2008), Islam et al. (2011), Malla & Banik (2015), Gosavi et al. (2020), and Mawa et al. (2022) for other catfish species. This study has divided gonad maturity into 5 stages for *P. atherinoides*.

**Ovary developmental stages (Figure 10):**

**Immature (Stage 1):** Ovaries are thin, colorless, and transparent. Small and filiform, they only occupy one-third of the abdominal space. Oocytes are exceedingly tiny and invisible to the naked eye.

**Maturing (Stage 2):** Ovaries are pale white/cream in color, with minor granulation discernible to the naked eye. Half of the abdominal cavity is taken up by enlargement signs. Small ova, visible with the naked eye, begin to appear.

**Mature (Stage 3):** Ovaries are whitish-yellow in color and have a greater anterior expansion than Stage 2. Two-thirds of the abdominal cavity is lobular. The ova have a distinct yolk.

**Ripe (Stage 4):** Yellowish, voluminous, and with blood vessels; occupies two-thirds of the abdominal cavity, with a loosening of the previously compact arrangement of ova.

**Spent (Stage 5):** Flaccid, wrinkled, displaying a smaller size than Stage 4; ovaries become drastically smaller in both length and weight and show a sac-like look with a pale white tint.

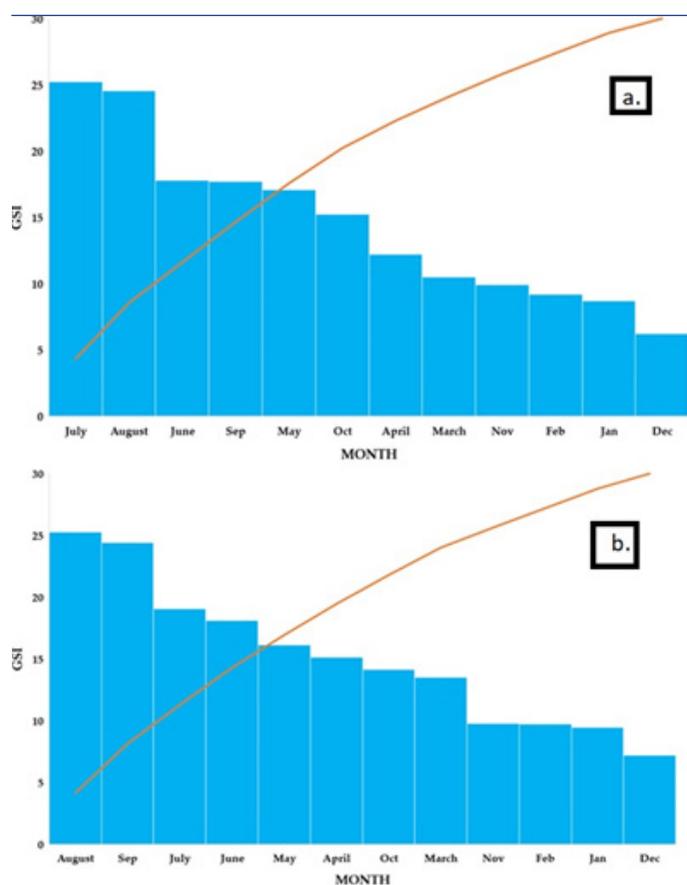
**Testis developmental stages (Figure 11):**

**Immature (Stage 1):** Testis are thin, threadlike, transparent, and white in color, with unorganized tubules.

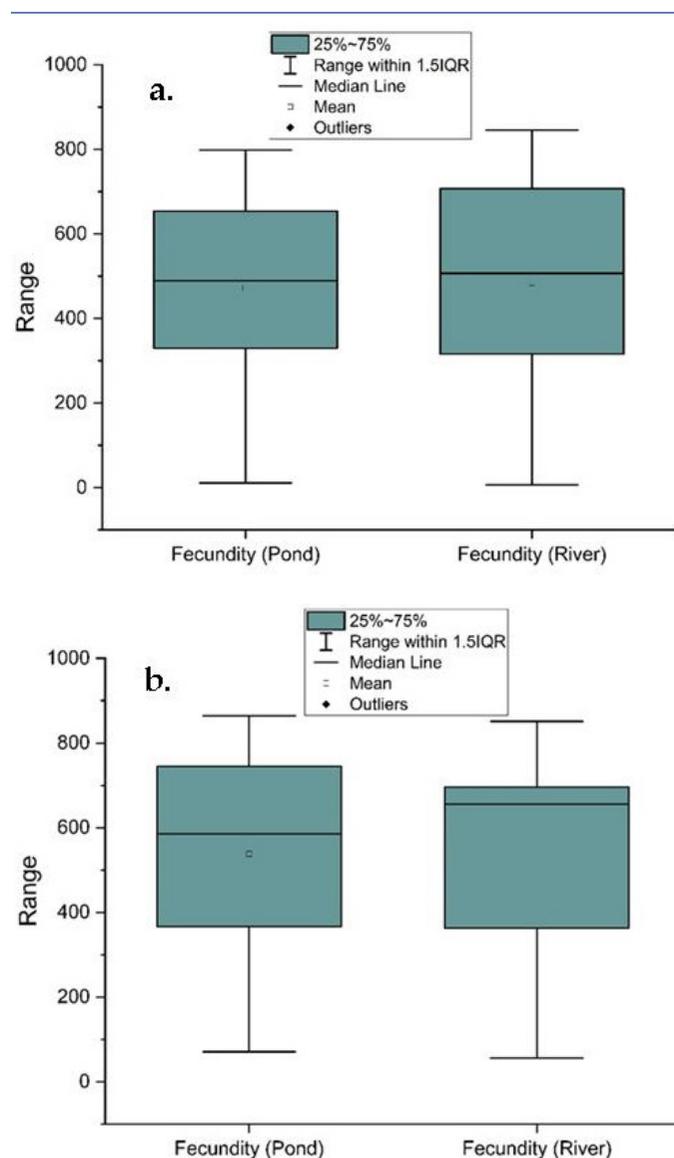
**Maturing (Stage 2):** Testis appear slightly longer and slightly more bulbous. The appearance of an indistinct vasa differentia.

**Mature (Stage 3):** Testis become large and creamy white in color. The Ductus difference is distinct. All spermatogenic cells with the maximum concentration of spermatids are present in seminiferous tubules.

**Ripe (Stage 4):** Testis are mature and more than two-thirds of the abdominal cavity in size. Spermatozoa-filled seminiferous tubules are easily discharged from the testis with a bit of pressure.



**Figure 6.** Monthly variation of GSI for *P. atherinoides* in Paschim Medinipur district's: a.) ponds; b.) rivers.

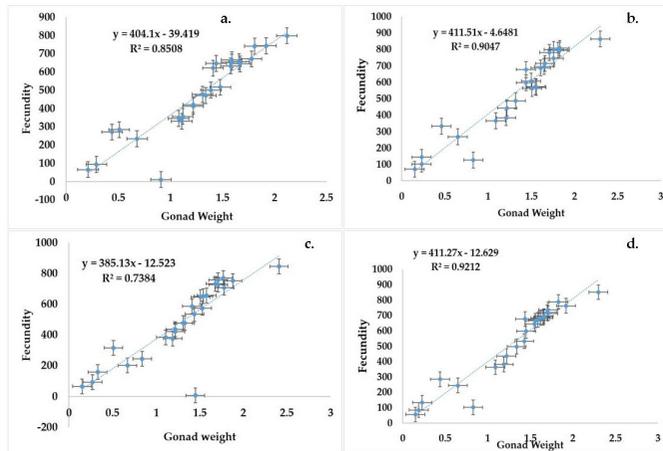


**Figure 7.** Fecundity of *P. atherinoides* in both habitats of (a) the Jhargram and (b) Paschim Medinipur districts.

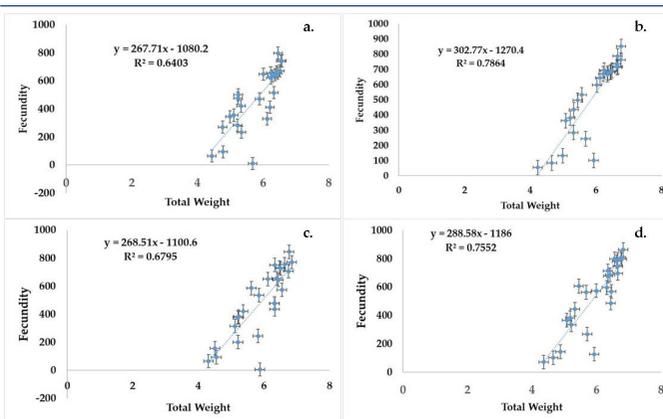
**Table 2.** Total weight, gonad weight, and fecundity of *P. atherinoides* in both habitats of the Jhargram and Paschim Medinipur districts

District	Total Weight(g)				Gonad weight (g)				Fecundity			
	Min	Max	M	SD	Min	Max	M	SD	Min	Max	M	SD
JP	4.44	6.57	5.79	0.659	0.21	2.12	1.264	0.50	65.0	798	482.7	203.02
JR	4.31	6.85	5.90	0.748	0.15	2.41	1.250	0.57	65.0	845	492.6	236.91
MP	4.23	6.77	5.93	0.712	0.15	2.30	1.311	0.56	56.0	851	526.9	243.32
MR	4.36	6.83	5.97	0.738	0.15	2.30	1.319	0.56	71.0	864	538.3	245.12

N = 104; JP = Jhargram Pond; JR = Jhargram River; MP = Paschim Medinipur Pond; MR = Paschim Medinipur River



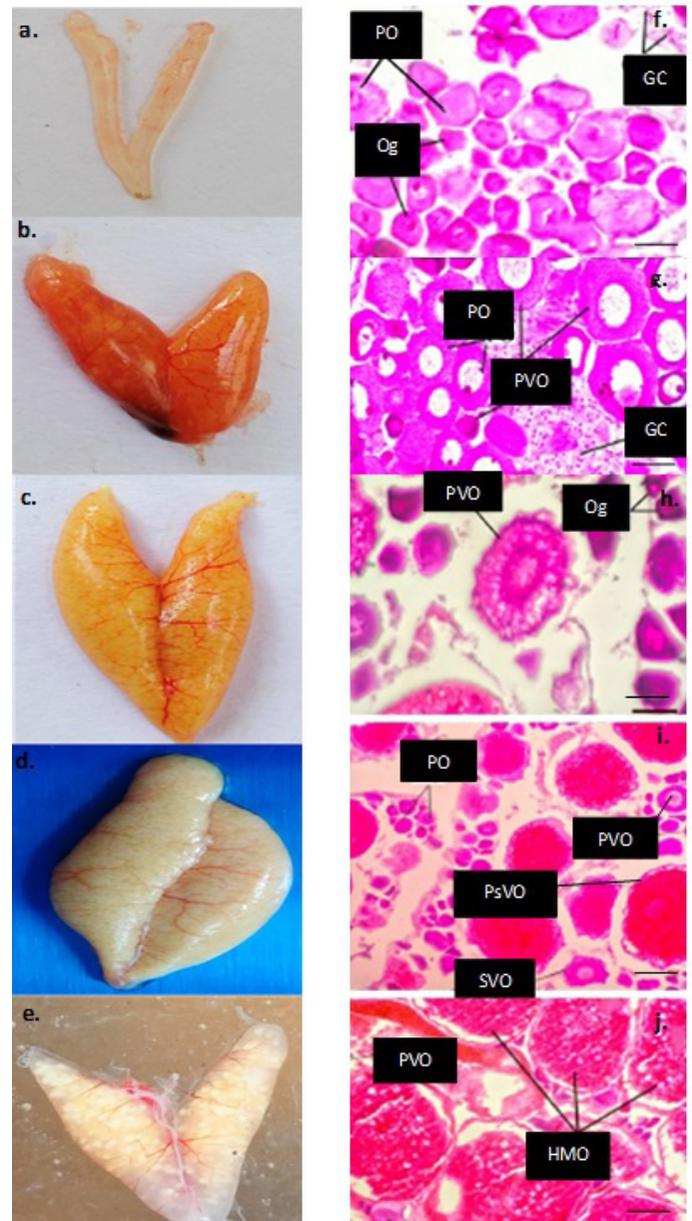
**Figure 8.** Relationship between fecundity and gonad weight for *P. atherinoides* in the Jhargram (a & b) and Paschim Medinipur (c & d) districts; a and c are ponds; b and d are rivers.



**Figure 9.** Relationship between fecundity and total weight for *P. atherinoides* in Jhargram (a & b) and Paschim Medinipur (c & d) districts; a and c are ponds; b and d are rivers

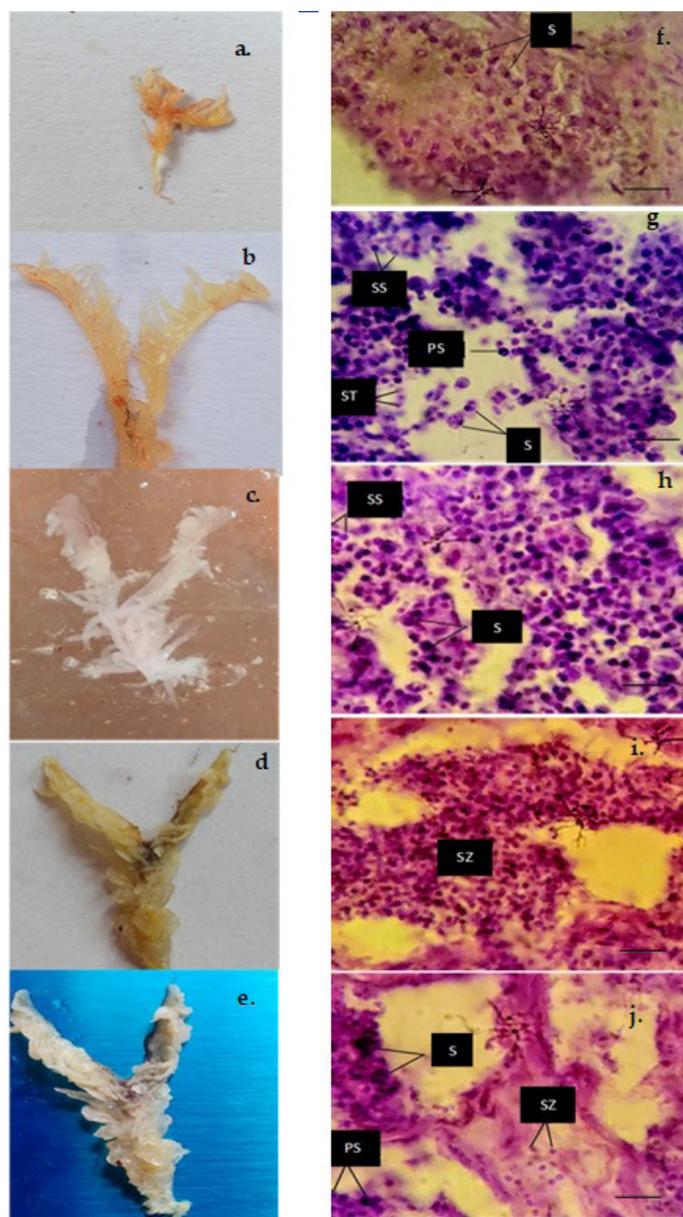
**Spent (Stage 5):** Flaccid gonads fill over half of the abdominal cavity in the spent (Stage 5) condition. Regression of the testis occurs. The cells seem to merge. High amount of primary and secondary spermatocytes.

During the study of ovary development in the lentic habitats (ponds), Stage 1 was noticed from November-March, with a peak in December and a minimum in May. Stage 2 was noticed from November-June, with a peak in April and a minimum in November. Stage 3 was noticed from March-September, with a peak in June and a minimum in September. Stage 4 was noticed from April-September, with a peak in July and a minimum in April. Stage 5 was noticed from June to November, with a peak in October and minimum in June. In the lotic habitats (rivers), Stage 2 of ovary development was noticed from November-July, with a peak in March and a minimum in July. Stage 4 was noticed from April-September, with a peak in July and a minimum in April. Stages 1, 3, and 5 were observed to have the same periods as those in lentic habitats (Figure 12).



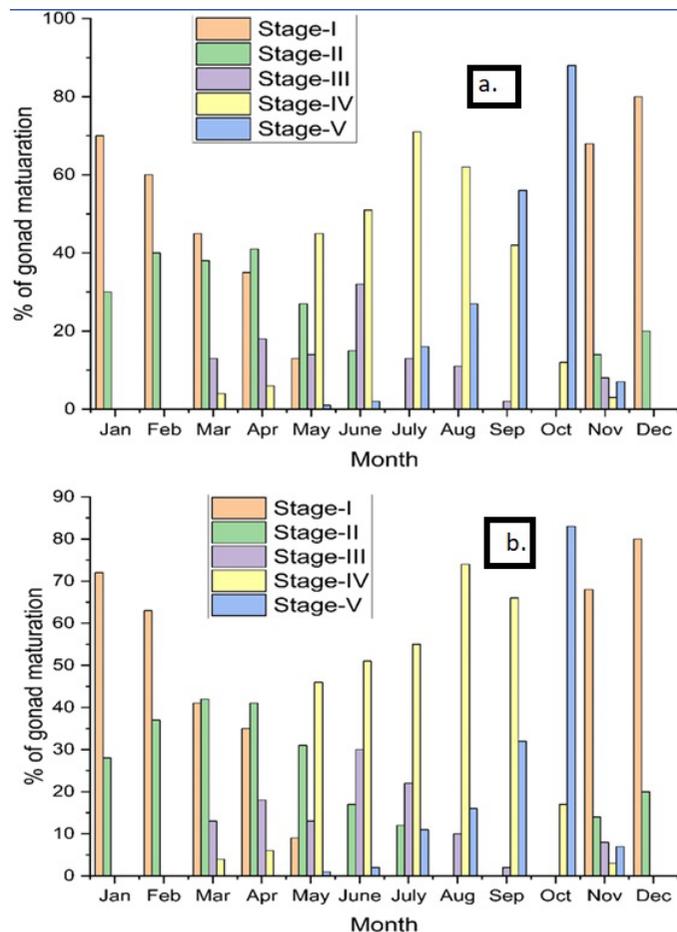
**Figure 10.** External (a-e) and microscopic (f-j) ovary of *P. atherinoides*. Stage 1 (a & f); Stage 2 (b & g); Stage 3 (c & h); Stage 4 (d & i); Stage 5 (e & j). GC = germ cells, Og = oogonia, PO = primary oocyte, PVO = primary vitellogenic oocyte, PsVO = post-vitellogenic oocyte, SVO = secondary vitellogenic oocyte, HMO = hydrated mature oocyte. Scale bar is at 100  $\mu$ m.

For the study of testis development in the lentic habitats, Stage 1 was noticed from November-May, with a peak in December and a minimum in May. Stage 2 was noticed from November-June, with a peak in April and a minimum in November. Stage 3 was noticed from February-December, with a peak in June and a minimum in December. Stage 4 was noticed from April-October, with its peak in July and minimum in April. Stage 5 was noticed from June-October, with a peak in October and minimum in June. For the lotic habitats (rivers), Stage 1 of testis



**Figure 11.** External (a-e) and microscopic (f-j) testes of *P. atherinoides*. Stage 1 (a & f); Stage 2 (b & g); Stage 3 (c & h); Stage 4 (d & i); Stage 5 (e & j). S = spermatogonia, PS = primary spermatocytes, SS = secondary spermatocytes, ST = spermatids, SZ = spermatozoa. Scale bar is at 100 µm.

development was noticed from November-May, with a peak in January and a minimum in May. Stage 2 was noticed from November-July, with the high in April and low in July. Stage 3 was noticed from February-December, with a peak in June and a minimum in February. Stage 4 was noticed from March-October, with the high in August and low in March. Stages 1, 3, and 5 were also observed to be similar to those in the pond habitats (Figure 13). The results for GSI, gonad development, and fecundity depict *P. atherinoides* to breed once a year between June-September.



**Figure 12.** Monthly ovary maturation stages of *P. atherinoides* in both districts: a.) ponds; b.) rivers.

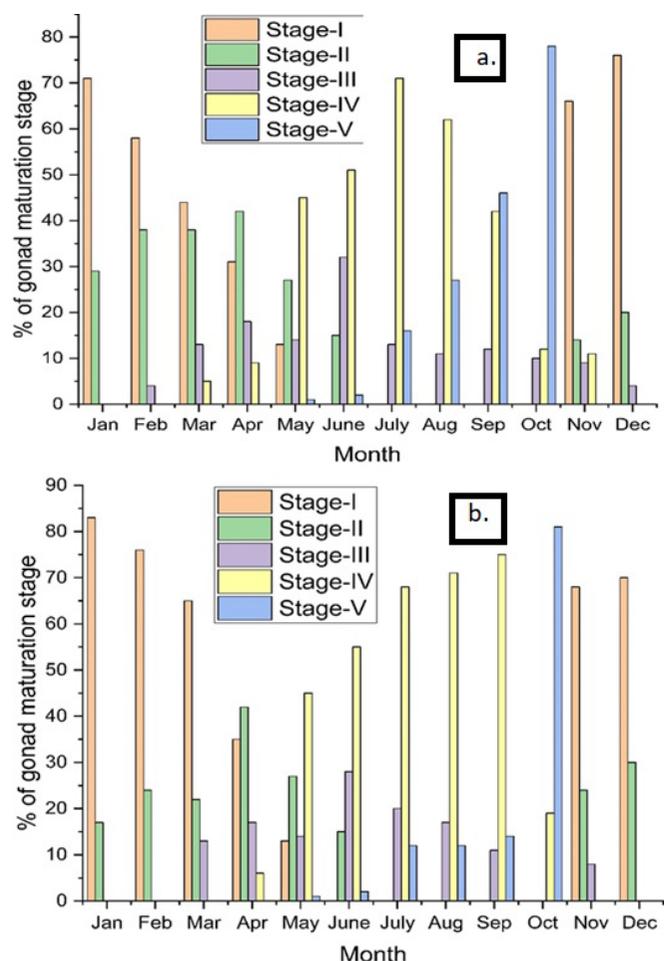
**CONCLUSION**

The breeding season of *P. atherinoides* has been determined in the study area to occur between June-September. Hence, the study suggests that government fishery managers take certain restrictions when capturing this species during their peak breeding season between June-September. The study's findings can be valuable tools for developing management and protection strategies for conservation and captive propagation. The knowledge from the current study may be applied for better managing *P. atherinoides* in India and for future conservation strategies, as well as for adopting this species as a possible candidate for commercial aquaculture.

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**Conflict of Interest:** The authors declare no conflicts of interest.

**Ethics Committee Approval:** Ethical approval was obtained from IAEC (Approval no. 18/IAEC (05)/RNLKWC/2019; dated: July 27, 2019).



**Figure 13.** Monthly testis maturation stages of *P. atherinoides* in both districts; a.) ponds; b.) rivers.

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# The Effect of Temperature on Growth and Odour Production in Three Cyanobacteria Species

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## ABSTRACT

Taste and odour episodes associated with geosmin and 2-methylisoborneol (MIB) produced by Cyanobacteria are common problems affecting drinking water supplies. However, it is difficult to find the source and species responsible for taste and odour production. The aim of this study was to investigate the effect of temperature, one of the most important environmental factors, on the growth and odour production of *Microcoleus* sp. MCAS-MC01 and compare the results with those of two other odour-producing cyanobacteria (*Oscillatoria* sp. UHCC 0332 and *Phormidium* sp. NIVA-CYA 7) isolated and kept in culture collections from geographically different areas. *Microcoleus* sp. MCAS-MC01 is a newly isolated geosmin and MIB producer from Türkiye. After complaints arose from consumers in a nearby city, samples were taken and a cyanobacterium, *Microcoleus* sp., was isolated from the samples and grown in Z8 medium. Experiments were conducted at 20°C and 25°C for 10 weeks, and the growth and MIB-geosmin concentrations of cultures were monitored weekly. The geosmin concentration reached a maximum 65.73 µg/l and the maximum MIB concentration was 626.65 µg/l in the study. Higher temperature had a significant positive effect on MIB levels in *Microcoleus* sp. MCAS-MC01 and geosmin levels in *Phormidium* sp. NIVA-CYA7 ( $p < 0.05$ ). On the other hand, the temperature did not affect the growth of all three strains ( $p > 0.05$ ). The results showed that taste and odour problems are species-specific, and in some species, they are stimulated by an increase in temperature. This study contributes to the understanding of taste and odour problems in relation to temperature.

**Keywords:** Cyanobacteria, Geosmin, 2-methylisoborneol, *Microcoleus*, *Oscillatoria*, *Phormidium*

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## INTRODUCTION

Climate change and eutrophication are a growing global issue and cause an increase in the occurrence of taste- and odour-producing cyanobacteria, which are particularly significant in drinking water reservoirs (Akcaalan et al. 2022; Mantzouki et al. 2018). Even if hazardous chemicals are not always the source of aquatic taste and odour, the drinking water sector suffers adverse effects as a result (Watson 2004), since customers consider taste and odour as the main measure of the safety of drinking water and complain at levels as low as 10 ng/l geosmin and MIB (Zamyadi et al. 2015). Geosmin and MIB are produced by members of certain

groups of benthic and pelagic aquatic microorganisms found in freshwater ecosystems (Watson & Jüttner 2019). Furthermore, there are a few additional biological sources that are often overlooked, especially those that originate from drinking water treatment plants and terrestrial ecosystems (Jüttner & Watson 2007).

Water utilities rarely monitor benthic cyanobacteria, although there is growing evidence that they represent a significant source of taste and odour problems (Gaget et al. 2022). Benthic cyanobacteria grow in different matrices such as sediments, biofilms, and floating mats, and they can detach and colonise in treatment plants (Gaget et al. 2020). However, a systematic under-



standing of how benthic cyanobacteria contribute to taste and odour episodes is still lacking (Gaget et al. 2022). To date, some benthic taste and odour-producing cyanobacteria from *Microcoleus*, *Oscillatoria*, *Phormidium*, *Heteroleibleinia*, *Leibleinia*, *Plectonema*, *Nostoc*, *Tychonema*, *Kamptonema*, *Jaaginema*, *Lyngbya*, *Leptolyngbya*, and *Symplocastrum* genera have been reported as geosmin or MIB producers (Watson et al. 2016). A total of 132 cyanobacterial strains from 21 genera and 72 cyanobacterial strains from 13 genera have been reported for geosmin and MIB production, respectively (Devi et al. 2021).

Consumer complaints are an important step in coping with taste and odour problems in drinking water. After increasing complaints about the taste and odour from a nearby city, samples were taken to measure geosmin and MIB in the drinking water reservoir. Some samples from benthic mats at the entrance of treatment plants were also taken, and the odour-producing cyanobacterium were isolated. The species was identified as *Microcoleus* sp. MCAS-MC01, which is the first isolate to produce MIB and geosmin in Türkiye. Furthermore, to reveal the effect of temperature on growth and taste and odour production, a study was conducted together with two different cyanobacteria strains (*Oscillatoria* sp. UHCC 0332 and *Phormidium* sp. NIVA-CYA 7) isolated from geographically different areas to understand the production of these metabolites in different cyanobacteria strains.

## MATERIALS AND METHODS

### Cyanobacteria strains, culture conditions, and sampling procedures

Three benthic filamentous cyanobacteria (*Microcoleus* sp., *Oscillatoria* sp. and *Phormidium* sp.) were used in this study. *Microcoleus* sp. MCAS-MC01 (Microalgae Culture Collection of Faculty of Aquatic Sciences, İstanbul University) was isolated from a drinking water treatment plant. The isolate was identified using molecular and morphological methods according to Strunecký et al. (2013). *Oscillatoria* sp. UHCC 0332 was obtained from the HAMBİ Microbial Culture Collection, University of Helsinki. It is a known geosmin and MIB producer. *Phormidium* sp. NIVA-CYA 7 was obtained from the Norwegian Culture Collection of Algae, NORCCA. It is a known geosmin producer. All strains were cultured in Z8 medium at 20°C and 25°C with a 12 h light:12 h dark period for 10 weeks, and sampling was performed at weekly intervals. The lower temperature was selected as 20°C because cyanobacterial dominance generally occurs at higher (>20°C) temperatures in aquatic ecosystems (Ozbayram et al. 2022; Robarts & Zohary, 1987). The higher selected temperature was 25°C because the benthic cyanobacteria growth rate is generally optimum around 25°C in natural ecosystems and the 5°C temperature change could have a different effect on different cyanobacterial species (Mantzouki et al. 2018). To homogenise the aggregated cultures before sampling, the cultures were sonicated from the 2nd week. The growth of the three cyanobacteria strains was estimated by measuring the optical density at 750 nm (OD750) (Ernst et al. 2005).

### Quantitative analysis of odour production

Geosmin and MIB analysed by Headspace-Solid Phase Microextraction (HS-SPME) coupled Gas Chromatography-Mass Spectrometry (GC-MS) according to a standard protocol (Kaloudis et al., 2016).

The GC oven temperature was started at 50°C and reached 250°C (12°C/min). Helium was used as the mobile phase (1 ml/min). The commercial standards of geosmin and MIB (Sigma CRM47525) were used for qualitative and quantitative analyses. Geosmin and MIB were quantified according to their m/z ratios (95 for MIB and 112 for geosmin) using the selected ion mode. Qualitative m/z values for ions were 112 and 126 for geosmin and 95, 108, and 135 for MIB.

### Statistics

The t-test in "rstatix" package (Kassambara, 2023) in R software was used to analyse the temperature effect on growth and odour (geosmin and MIB) concentration at a 95% confidence level (ver. 4.3.1) (R Core Team 2024). Spearman correlation with the level of significance as  $p < 0.001$ ,  $p < 0.05$ , and  $p < 0.01$  was used to analyse the correlations between growth and odour concentration (geosmin and MIB) in Jamovi software (ver.2.3.1) (Jamovi project 2022). One-way ANOVA analysis was conducted to analyse temporal variance of geosmin and MIB concentrations at a 99% confidence level in Jamovi.

## RESULTS AND DISCUSSION

Odour production was observed in all three cyanobacteria species for 10 weeks at different concentrations. The temperature had no statistically significant effect on growth ( $p > 0.05$ ) indicating these two temperatures (20°C and 25°C) did not have a dramatic effect on the growth of the three cyanobacteria strains.

It was observed that the cultures generally started to lose their colour after the 9<sup>th</sup> week of the experiment (Figure 1). *Microcoleus* sp. MCAS-MC01 and *Oscillatoria* sp. UHCC 0332 cultures started to die earlier at 20°C than at 25°C. On the other hand, *Phormidium* sp. NIVA-CYA 7 culture started to lose its colour earlier at 25°C than at 20°C (Figure 1).

Our results showed that temperature had a significant positive

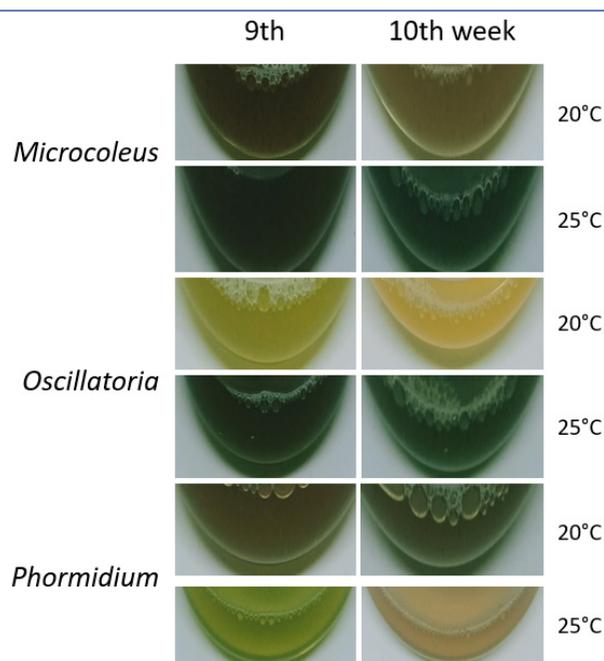


Figure 1. Colour changes in cultures.

effect on MIB production in *Microcoleus* sp. ( $t_{(11.85)}=-2.82, p<0.05$ ) and geosmin production in *Phormidium* sp. ( $t_{(10.47)}=-2.19, p<0.05$ ). However, there was no significant effect on geosmin production in the other two strains and MIB production in *Oscillatoria* sp. ( $p>0.05$ ) (Figure 2).

The maximum geosmin concentration was detected in the 8<sup>th</sup> week at both temperatures reaching 63.11 µg/l at 20°C (Figure 2a) and 65.73 µg/l at 25°C in *Microcoleus* sp. (Figure 2b). Geosmin production followed an increasing trend until the 8<sup>th</sup> week and started to decrease afterwards (Figure 2). The optical density values increased at both temperatures during the experiment. Although geosmin production did not differ with temperature, the geosmin concentration of *Microcoleus* sp. MCAS-MC01 culture was dramatically higher than the threshold limits (10 ng/l) and reported environmental concentrations (Qiu et al. 2023). *Microcoleus* species are ubiquitous and form thick mats in different habitats, including freshwater ecosystems, a street flowerbed in an urbanised area, garden soil, and aquaculture areas (Tee et al. 2021; Churro et al. 2020; Alghanmi et al. 2018). For example, an episodic taste and odour problem related to the geosmin producer *Microcoleus* sp. was reported in a trout farming area, which was linked to the appearance of earthy/musty off-flavour and poor water quality during the water recirculation period (Robin et al. 2006). However, there are a limited number of isolated and identified geosmin/MIB-producing *Microcoleus* species: *Microcoleus pseudautumnalis* and *M. autumnalis* (formerly *Phormidium autumnale*) from Japan, *Microcoleus vaginatus* CCALA 145 (formerly *Phormidium autumnale*) from Switzerland, *Microcoleus vaginatus* from Iraq, *M. asticus* from Portugal and *Microcoleus* sp. from USA (Teneva et al. 2023; Churro et al. 2020; Niiyama & Tuji, 2019; Alghanmi et al. 2018; Oikawa et al. 2015; Izaguirre & Taylor, 1995). Therefore, our newly isolated geosmin and MIB producer strain from Türkiye makes a major contribution to research on taste and odour producers from *Microcoleus* genus, demonstrating a wide geographical distribution.

MIB levels at 25°C were significantly higher than those at 20°C in *Microcoleus* sp. ( $t_{(11.85)}=-2.82, p<0.05$ ) reaching 626.65 µg/l at 25

°C in the 5<sup>th</sup> week (Figure 3b) and 320.48 µg/l at 20°C in the 8<sup>th</sup> week (Figure 3a). MIB production increased until the 8<sup>th</sup> and 5<sup>th</sup> weeks at 20°C and 25°C, respectively, and started to decrease in the following weeks (Figure 3). In line with our result, Alghanmi et al. (2018) also found maximum MIB concentrations at 25°C in *Microcoleus vaginatus* compared with 10°C and 33°C. Similarly, *Microcoleus autumnalis* (formerly *P.autumnale*) produces more MIB at high water temperatures above 20°C (Oikawa et al. 2015). In our study, the maximum total MIB level of our strain (626.65 µg/l) was significantly higher than the MIB concentrations measured in *M. vaginatus* (135.8 ng/l) and *Lyngbya* sp. (260 µg/l) (Izaguirre & Taylor, 1995). The results show that our isolate, *Microcoleus* sp., can respond to increasing temperatures by producing higher concentrations of the odour compound (MIB) and by surviving longer in the environment. Our strain is isolated from a water treatment plant that supplies drinking water to more than 200.000 people. These results are critical for determining water quality management strategies to be implemented in water treatment plants. Water authorities may encounter the problem intermittently in their drinking water systems, especially in summer months, and it may be necessary to implement appropriate treatment systems, such as ozonation or activated carbon, to be used when odour episodes occur (Oikawa et al. 2015).

Temperature had no significant effect on geosmin and MIB production in *Oscillatoria* sp. ( $p>0.05$ ). The maximum geosmin production was 125.86 µg/l at 25°C and 90.8 µg/l at 20°C in the 9<sup>th</sup> week, followed by a significant decrease in the geosmin concentrations at both temperatures (Figure 4). Geosmin concentration increased from the 4<sup>th</sup> week at 20°C until the 9<sup>th</sup> week, and following this, the level of geosmin started to decrease (Figure 4a), whereas at 25°C, the trend over time was not as pronounced (Figure 4b).

On the contrary, maximum MIB concentrations were significantly higher than geosmin at both temperatures and measured as 1339 µg/l at 25 °C (9<sup>th</sup> week) and 768.69 µg/l at 20°C (8<sup>th</sup> week) (Figure 5). A significant MIB increase was observed at 20°C from the 4<sup>th</sup> week until the 8<sup>th</sup> week, following which the level of MIB

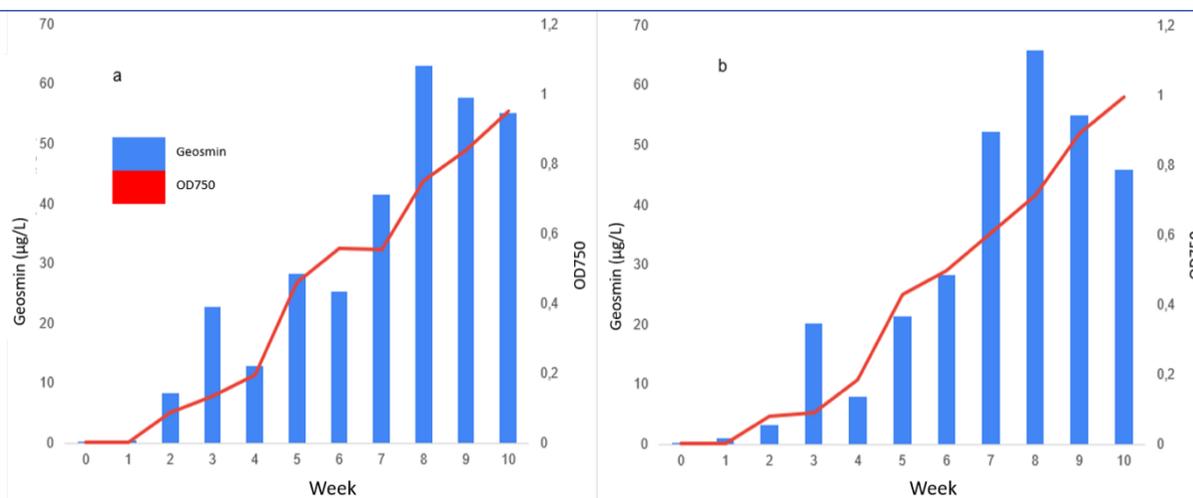
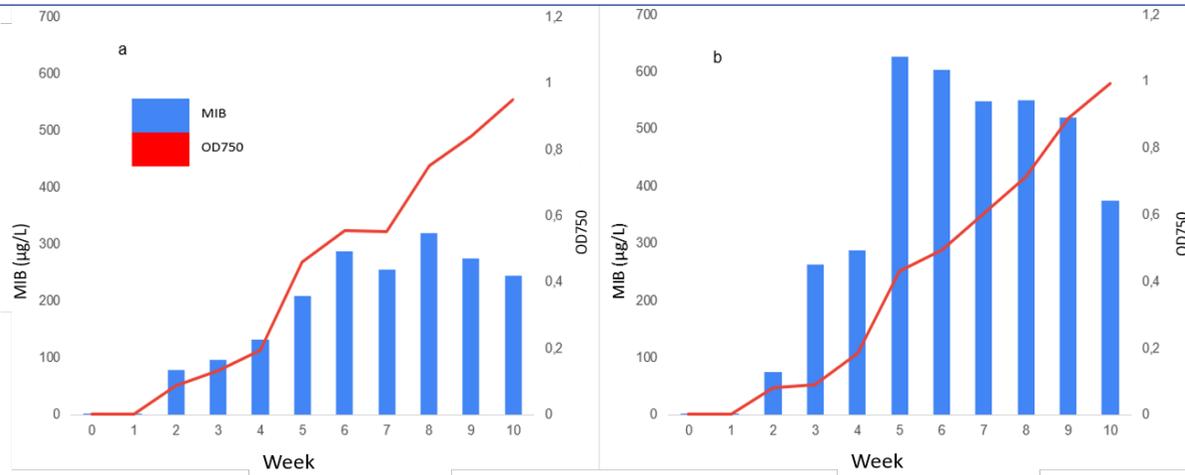
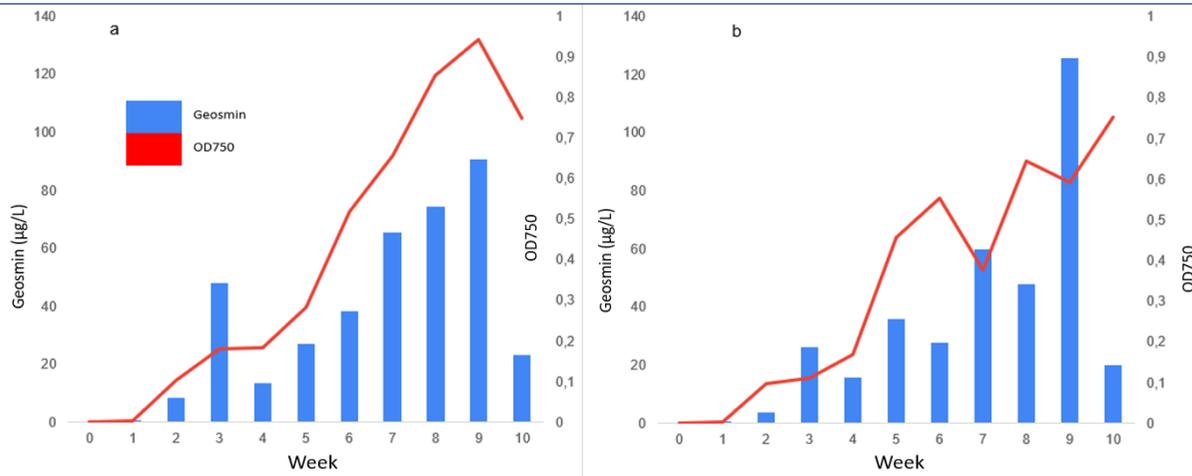


Figure 2. Comparison of geosmin production and growth in *Microcoleus* sp. at a) 20°C and b) 25°C.



**Figure 3.** Comparison of MIB production and growth in *Microcoleus* sp. at a) 20°C and b) 25°C.



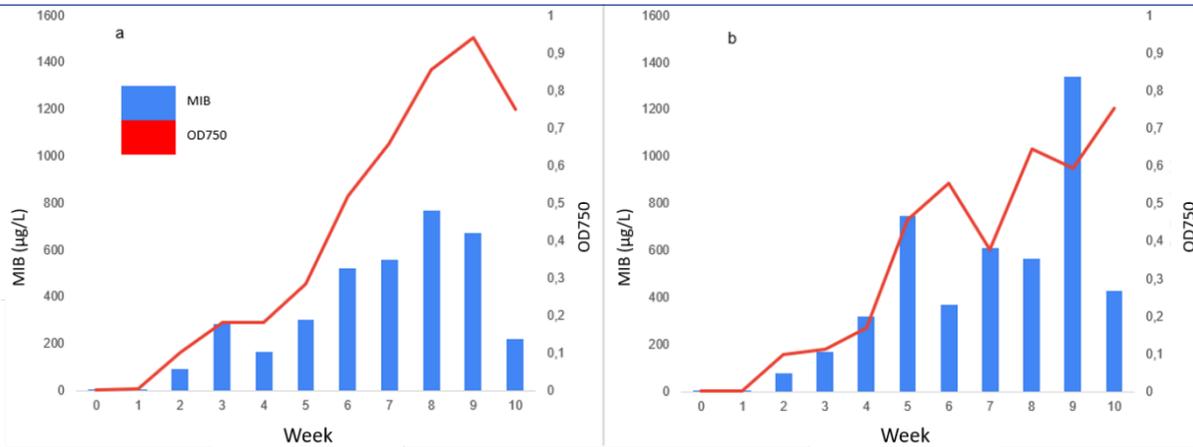
**Figure 4.** Comparison of geosmin production and growth in *Oscillatoria* sp. at a) 20°C and b) 25°C.

started to decrease (Figure 5a). Although there was not a distinct MIB trend at 25°C over time (Figure 5b), *Oscillatoria* sp. showed better growth at that temperature, consistent with the literature (Sivonen 1990, Cai et al. 2017). Chu et al. (2007) found that *Oscillatoria* sp. became dominant over the common cyanobacterium *Microcystis* sp. at temperatures below 25°C, whereas the contrary occurred at temperatures of 30°C and above. When there is competition between these two cyanobacteria species in natural ecosystems, temperature could be an important driving factor, and *Oscillatoria* sp., an earthy and mouldy odour producer, may have an advantage over *Microcystis* sp., which does not produce such odours, at 25°C and below. The information observed in the experimental studies also supports our understanding of the odour production dynamics in aquatic ecosystems.

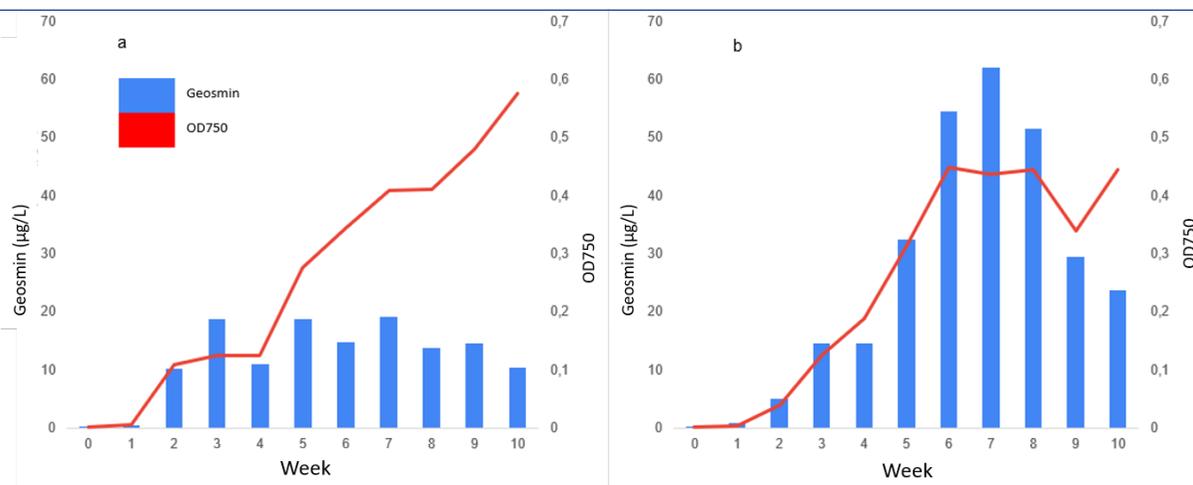
The concentrations of geosmin and MIB in *Oscillatoria* sp. varied greatly at both temperatures. Although these metabolites utilise common metabolic pathways in cyanobacteria, their production levels may differ (Jüttner & Watson, 2007). However, since the removal of MIB from water is more difficult compared with geosmin, high levels of MIB can cause a very high economic loss for

treatment plants (Zamyadi et al., 2015). The temperature could trigger odour production in various cyanobacteria species. Our results showed that *Oscillatoria* sp. produced both metabolites in higher concentrations at 25°C than at 20°C. In line with our results, Cai et al. (2017) also found that the maximum geosmin production in *Oscillatoria limosa* occurred at 25°C.

Higher temperatures resulted in higher geosmin production in *Phormidium* sp. (Figure 6). The maximum geosmin production was measured at 25°C as 62.06 µg/l in the 7th week (Figure 6b). Our results are in line with *Phormidium amoenum* isolate from South Australia, which had a maximum geosmin concentration measured as 49 µg/l at 25°C (Li et al. 2012). Additionally, *Dolichospermum smithii* could also produce a maximum total geosmin concentration of 19.82 µg/l at 25°C (Shen et al. 2022). In our study, the maximum geosmin production at 25°C was three times higher than that at 20°C (19.02 µg/l at 20°C, 62.06 µg/l at 25°C) in *Phormidium* sp. The geosmin concentration started to increase from the 5th week until the 7th week and started to decrease the following weeks at 25°C (Figure 6b). On the other hand, there was no significant change over time, with maximum production



**Figure 5.** Comparison of MIB production and growth in *Oscillatoria* sp. at a) 20°C and b) 25°C.



**Figure 6.** Comparison of geosmin production and growth in *Phormidium* sp. at a) 20°C and b) 25°C.

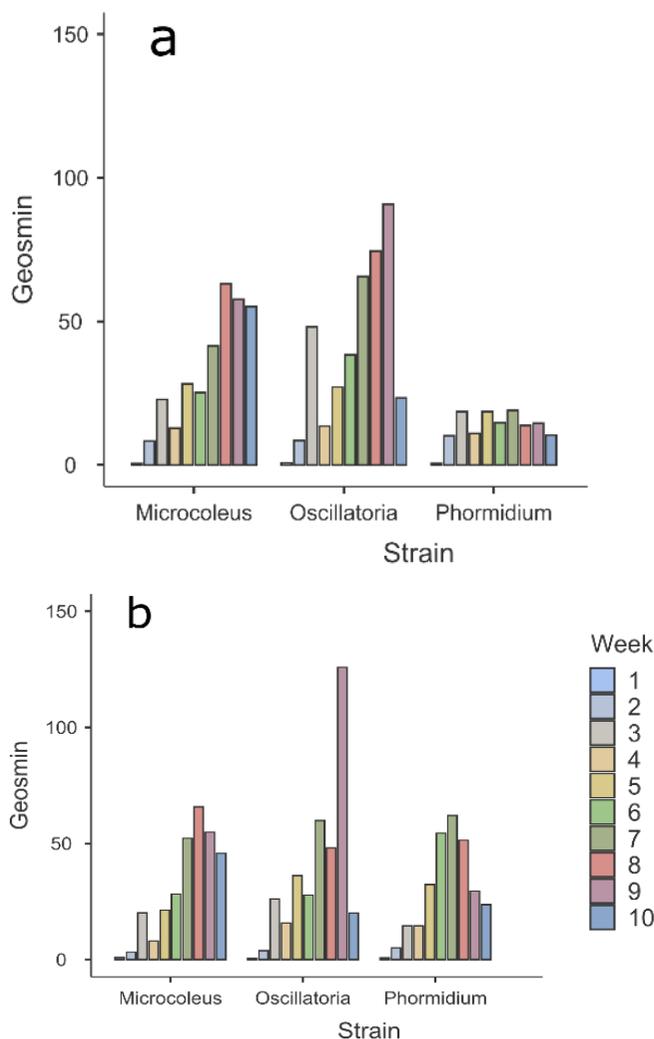
at week 7 being slightly higher than in other weeks at 20°C (Figure 6a). However, the growth curve of the strain maintained at 20°C continued to increase throughout the experiment. Fujimoto et al. (1997) found that *Phormidium* sp. could become dominant over *Microcystis* sp. at 20°C and a high N:P ratio, whereas the advantage shifted to *Microcystis* sp. at 25°C. Therefore, the competitive advantage of *Phormidium* sp. and associated odour production at relatively lower temperatures in aquatic environments should be considered in water quality management.

These three odour-producing cyanobacteria, *Microcoleus* sp. MCAS-MC01, *Oscillatoria* sp. UHCC 0332 and *Phormidium* sp. NIVA-CYA 7, showed similar thermotolerance profiles at 20°C and 25°C. Comparing the total geosmin production capacity, *Oscillatoria* sp. UHCC 0332 showed highest capacity reaching up 125.86 µg/l, *Microcoleus* sp. MCAS-MC01 reaching up 63.11 µg/l, and *Phormidium* sp. NIVA-CYA 7 to 62.06 µg/l (Figure 7). Regarding MIB production capacity, *Oscillatoria* sp. UHCC 0332 showed higher capacity by reaching up 1339 µg/l in comparison to the highest concentration measured as 626.65 µg/l in *Microcoleus* sp. (Figure 8).

Geosmin variation was significant over time in *Microcoleus* sp., *Oscillatoria* sp., and *Phormidium* sp. at both 20°C and 25°C (Figure 7). Comparing the temporal variation of geosmin, the concentration was significantly different for each week at both 20°C and 25°C in all strains except for *Phormidium* sp. (Figure 7).

Comparing temporal variation of MIB concentration, each week was significantly different in *Microcoleus* sp. and *Oscillatoria* sp. at both 20°C and 25°C (Figure 8).

Geosmin had a strong positive correlation with growth in *Microcoleus* sp. at both temperatures ( $r = 0.903$  for 20°C and  $r = 0.842$  for 25°C respectively,  $p < 0.001$ ), while MIB had only a strong correlation with growth at 20°C ( $r = 0.840$ ,  $p < 0.001$ ). At 25°C, a moderate positive correlation between MIB and growth ( $r = 0.588$ ,  $p < 0.05$ ) was detected. Similarly, both geosmin and MIB had a strong positive correlation with growth in *Oscillatoria* sp. at both temperatures ( $r = 0.794$ ,  $p < 0.01$  for geosmin at 20°C and  $r = 0.612$ ,  $p < 0.05$  at 25°C;  $r = 0.842$ ,  $p < 0.01$  for MIB at 20°C and  $r = 0.721$ ,  $p < 0.05$  at 25°C) (Figure 4 and Figure 5), which was consistent with the results of previous studies (Shen et al. 2022, Jüttner & Watson 2007). Shen et al. (2022) highlighted that tem-

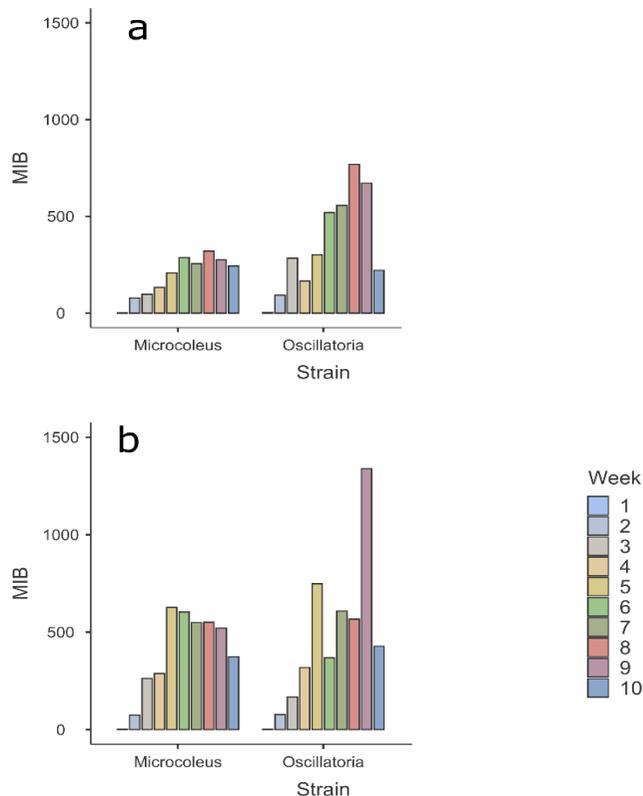


**Figure 7.** Comparison of total geosmin concentration in three strains (a. 20°C, b. 25°C).

perature can increase the potential of geosmin/MIB synthesis. We also found 20°C - 25°C temperature range is generally favourable for growth and odour production in cyanobacteria. Geosmin had no correlation with growth in *Phormidium* sp. at 20°C ( $r = 0.274$   $p > 0.05$ ) (Figure 6a), whereas it had a strong correlation at 25°C ( $r = 0.851$   $p < 0.001$ ) (Figure 6b). We can conclude that geosmin production in *Phormidium* sp. at 20°C may be suppressed because of a possible competition for the essential common substrates with Chl-a synthesis, as we found growth continued while geosmin concentration had a generally stationary trend at 20°C (Shen et al. 2021; Wang & Li, 2015).

## CONCLUSION

Our results highlight the important role of the strain-specific response to temperature changes. Furthermore, the production of geosmin and MIB compounds differed under different temperatures in our strain *Microcoleus* sp. MCAS-MC01 and *Phormidium* sp. NIVA CYA7. We found that a 5°C temperature difference had a dramatic effect on the odour production of different cyanobacterial strains. The total



**Figure 8.** Comparison of total MIB concentration in three strains (a. 20°C, b. 25°C).

odour concentrations without comparing intra- and extracellular components were measured in this study. Further studies are needed to address whether the temperature had a mechanism to increase intracellular odour production or to affect the release of odour compounds outside the cell. In addition to *in vitro* studies, to predict the effects of changing environmental conditions on odour production in natural ecosystems, it is also important to determine the levels of odour compounds using *in situ* studies, such as mesocosm experiments to analyse other biotic and abiotic triggers.

**Conflict of Interest:** The author has no conflicts of interest to declare.

**Ethics committee approval:** Ethics committee approval is not required.

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# Phenotypic and Genotypic Antibiotic Resistance of *Staphylococcus warneri* and *Staphylococcus pasteurii* Isolated from Stuffed Mussels

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## ABSTRACT

This study aimed to assess the presence of *Staphylococcus* species in stuffed mussel samples sold in Çanakkale province and to determine the antibiotic resistance of the isolates. A total of 246 stuffed mussel samples were examined, and two different *Staphylococcus* (*S. warneri* and *S. pasteurii*) were isolated from 12.19% of the samples. Among the *Staphylococcus* isolates, *S. warneri* was detected at a rate of 73.33% and *S. pasteurii* at 26.67%. Bacterial isolates (N=30) were examined for their resistance to amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg), clindamycin (10 µg), vancomycin (30 µg), oxacillin (5 µg), tetracycline (30 µg), doxycycline hydrochloride (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), meropenem (10 µg), imipenem (10 µg), sulfamethox/ trimethoprim (25 µg), cefotaxime (30 µg), cephalothin (30 µg), ceftriaxone (30 µg), and levofloxacin (5 µg) antibiotics. Resistance to amoxicillin/clavulanic acid and erythromycin was found in all *S. warneri* isolates. In addition, all *S. pasteurii* isolates were found to be resistant to amoxicillin/clavulanic acid. In *S. warneri* isolates, at least three resistance genes (*BlaTEM*, *tetB-6*, *tetK-8*) and up to eight resistance genes (*BlaTEM*, *tetB-6*, *tetK-8*, *strA-strB*, *aphAI-IAB*, *ermC*) were identified. All *S. pasteurii* isolates exhibited *blaTEM*, *strA-strB*, and *aphAI-IAB* resistance genes. In conclusion, it was determined that antibiotic-resistant *S. warneri* and/or *S. pasteurii* contaminates stuffed mussel samples. This study will serve as a valuable resource for enhancing monitoring strategies in stuffed mussel production. Further studies should be conducted to determine whether the products are suitable for food safety. Therefore, it is necessary to clarify the disease effects and mechanisms of the pathogens identified in stuffed mussels.

**Keywords:** Stuffed mussel, *Mytilus galloprovincialis*, *S. warneri*, *S. pasteurii*, Public health, antibiotic resistance

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## INTRODUCTION

Consumers directly consume ready-to-eat foods without requiring processing, such as heat treatment (WHO, 1996; Mosupye & von Holy, 1999). Those prepared by street vendors and sold especially on the streets or other public places are also defined as ready-to-eat foods and beverages (FAO, 1997). Ready-to-eat meals are encountered in various presentations and packaging in markets, restaurants, and many other areas, with street foods being the most prevalent. Street

foods play a significant role in meeting a substantial part of our daily meal needs and are preferred by everyone, regardless of age or profession. Street foods can be classified as seafood, animal-derived products, pastries, sweets, fruits, and other gastronomic products (Demir et al., 2017). Stuffed mussels, especially in coastal cities, are among the most consumed street foods. Ready-to-eat foods are preferred for reasons such as easy accessibility, quick preparation, affordability, and compatibility with different taste preferences.



The most significant danger regarding street food is the uncertainty regarding its suitability for consumption in terms of food safety. The safety of foods depends on various common factors, ranging from the quality of raw materials to food processing and storage practices. Most vendors may expose the meals to conditions that are not suitable for food safety, such as "cross-contamination, unhygienic storage, and poor time-temperature" conditions (Lucca and Torres, 2006; Mepba et al., 2007). Products produced and sold without considering food safety can lead to situations that endanger human health. Bacteria that contaminate food due to various contamination sources and cause epidemic diseases in humans are among the primary concerns. In addition to causing various infections and diseases, these bacteria are becoming increasingly resistant to antibiotics, leading to challenging-to-treat illnesses. The World Health Organisation (WHO, 2017) has declared antibiotic-resistant bacteria as the "greatest and most urgent global threat" because as resistance to antibiotics increases, bacteria develop multiple antibiotic resistance. Therefore, antibiotics used for treatment do not yield successful results.

Stuffed mussels, generally prepared using *Mytilus galloprovincialis*, are ready-to-eat meals that are commonly sold on the streets in countries with coastlines along Türkiye and the Mediterranean. Throughout the processing, preparation, and cooking of stuffed mussels, they can be susceptible to human-induced contamination, and during the sales process, they may be exposed to environmental factors such as air pollution and insects. The high pH and nutrient profile of stuffed mussels create a favourable environment for microbial growth. Street vendors typically sell stuffed mussels in open-air environments on tables for 6-8 hours or even longer (Kışla and Üzgün 2008). Consumers use the mussel shell itself as a spoon, remove the stuffing, and consume it. In this case, if the mussel shell is contaminated, it becomes a source of risk when it comes into contact with the mouth. Leftover stuffed mussels at the end of the day are sometimes stored inappropriately in refrigerators (+4 °C), often at unsuitable ambient temperatures, to be put up for sale again the next day.

Studies conducted in Türkiye have reported that stuffed mussels may contain *Escherichia coli*, *Staphylococcus spp.*, *Bacillus cereus*, *Vibrio alginolyticus*, *Listeria monocytogenes*, yeast, mold, and anaerobic bacteria (Bingol et al., 2008; Durgun, 2013; Üzgün, 2015; Karademir, 2018; Güngörür, 2019). However, to the best of our knowledge, there is no detailed study examining the phenotypic and genotypic antibiotic resistances of *S. warneri* and/or *S. pasteurii* species isolated from stuffed mussels. *Staphylococcus warneri* has been isolated from various sources, including fermented foods, humans, and numerous animal species (Becker et al., 2014). Studies in the literature have reported the isolation of *S. warneri* from sea cucumbers (Kim et al., 2017) and marine fish flesh (Regecova et al., 2014). *Staphylococcus pasteurii* has been reported to be present in various foods, including goat milk (Chesneau et al., 1993), Italian sausages (Rantsiou et al., 2005), and retail beef (Bhargava et al., 2014), as well as in drinking water (Faria et al., 2009). However, no literature is available regarding the isolation of *S. warneri* and *S. pasteurii* from ready-to-consume stuffed mussels.

*S. pasteurii* (Petti et al., 2008; Savini et al., 2008; Ramnarain et al., 2019; Morfin-Otero et al., 2012; Sánchez et al., 2013; Savini et al., 2009a; Savini et al., 2009b) and *S. warneri* (Incani et al., 2010; Dimiatriadi et al., 2014; Gelman et al., 2022; Hoque et al., 2023a; Hoque et al., 2023b; Louail et al., 2023; Si et al., 2024) were appeared in reports of different diseases that could be associated with human. This study aims to investigate the presence of *S. warneri* and *S. pasteurii*, which are known to cause significant health problems in the respiratory, skin, digestive systems, etc., in humans, in stuffed mussels sold in Çanakkale and their resistance to antibiotics.

## MATERIALS AND METHODS

### Materials

In the scope of this study, stuffed mussels offered for sale in open and closed spaces in the market of Çanakkale Province were used as the material. Mussel samples (N=246) were collected from a total of 20 locations, with 10 from open spaces (street vendors) and 10 from closed spaces (restaurants, etc.), during the peak consumption seasons of spring (May), summer (June to August), and autumn (September-October) (Table 1). Ready-to-consume stuffed mussel samples were transported to the microbiology laboratory within 30 min in styrofoam boxes at +4°C.

### Methods

#### Isolation and identification of bacteria

For microbiological analyses, 10 g of mussel samples were homogenised in 90 ml of peptone water for 1 min. Decimal dilutions ( $10^{-1}$  to  $10^{-6}$ ) were prepared from the homogenate. From these dilutions, inoculations were made using the spread plate and pour plate methods. Mannitol Salt Phenol Red Agar (Merck 105404) was used as the culture medium. The culture plates were incubated 35°C for 3 days in the incubator to allow for the development of microorganisms. After the bacteria were purified from the Mannitol Salt Agar medium where they grew, classical methods (colony type, morphology, gramme +/- characteristics, oxidase, catalase,  $H_2S$ , indole, etc.) were used for genus-level identification. The isolates were grown for stocks in Brain Heart Infusion (Merck 110493) liquid medium. Subsequently, they were stored at -80°C in cryogenic tubes containing 30% glycerol until further molecular identification and antibiotic susceptibility testing were performed.

#### Species identification of the bacterial isolates

For DNA (Deoxyribonucleic acid) isolation, the EurX GeneMATRIX Bacterial & Yeast DNA isolation kit from Poland was utilised

**Table 1.** Sampling plan for the study.

Season/ Year	Locations/ Count	Count of the Sample
Spring/2021	Street vendors/2	15
Spring/2021	Restaurants/2	15
Summer/2021	Street vendors/5	60
Summer/2021	Restaurants/5	60
Autumn/2021	Street vendors/3	48
Autumn/2021	Restaurants/3	48

(<https://eurx.com.pl/docs/manuals/en/e3580.pdf>). The quantity and purity of the obtained DNA after isolation were assessed using the Scientific Nanodrop 2000 device (USA) through spectrophotometric measurements conducted at Thermo.

In the PCR (Polymerase Chain Reaction) study, target gene regions for species identification were amplified using the universal primers 27F–1492R. The primer sequences used were 27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGY-TACCTTGTTACGACTT 3' (Lane, 1991). The PCR mixture (35 µL) included approximately 1 PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 100 ng template DNA, 0.3 µM of each primer, and nuclease-free water. PCR amplification was performed using a thermal cycler (Kyratec, Geumcheon gu, Seoul Korea) with the following parameters: 95°C for 5 min (initial denaturation), 30 cycles of 95°C for 45 s (denaturation), 57°C for 45 s (annealing), 72°C for 60 s (extension), and a final step at 72°C for 5 min (final extension). Then, 10 µl of the PCR sample was loaded on a 1.5% agarose gel in 1 TAE containing ethidium bromide, and electrophoresis was performed for 90 min at 100 V. PCR products were cleaned and sequenced by BM Labosis (Ankara, Turkey) using the universal primers 27F–1492R. Sequence editing was performed using BioEdit (Bioedit v7.0.0). 16S sequences were compared against all GenBank *S. warneri* and *S. pasteurii* sequences using the BLASTN search at <http://blast.ncbi.nlm.nih.gov/>.

#### Antibiogram tests for *Staphylococcus* isolates

The Kirby-Bauer disc diffusion test was employed to determine the antibiotic resistance of the bacteria (Bauer et al., 1966). The analysis execution and interpretation of test results adhered to the standards set by the Clinical and Laboratory Standards Institute (CLSI, 2015; CLSI, 2017). The bacterial strains stored in the freezer were initially cultured at least twice in tryptic soy (TS) medium. Subsequently, the bacterial isolates were transferred to Mueller-Hinton (MH) solid medium. After successful growth, colonies displaying the best development were selected, and their density was adjusted to 0.5 McFarland in liquid medium (MH). From the adjusted-density liquid medium (0.5 McF), bacterial transfers were made to an appropriate solid culture medium using a sterile cotton swab. After bacterial inoculation on solid culture media, antibiotic discs [amoxicillin/clavulanic acid (30 µg), ampicillin (10 µg), erythromycin (15 µg), clindamycin (10 µg), vancomycin (30 µg), oxacillin (5 µg), tetracycline (30 µg), doxycycline hydrochloride (30 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), meropenem (10 µg), imipenem (10 µg), sulfamethox/ trimethoprim (25 µg), cefotaxime (30 µg), cephalothin (30 µg), ceftriaxone (30 µg), and levofloxacin (5 µg) were placed on the culture medium for the disc diffusion test. The plates were then incubated at 35°C for 24 h. The resulting zone diameters were measured using a ruler and interpreted according to the CLSI standards (CLSI, 2015; CLSI, 2017).

#### Identification of antibiotic resistance genes in bacterial isolates

For the analysis of bacterial antibiotic resistance genes, the EurX GeneMATRIX DNA Isolation Kit from Poland was used to isolate DNA from 30 samples. After DNA isolation, the quantity and purity of the obtained DNA were assessed through spectrophotometric measurements using a Thermo Scientific Nanodrop 2000

device (USA). The contents of the PCR cocktail were adjusted to 20 µL as 1 PCR buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.5 µM of each forward and reverse primer, 2 U Taq polymerase, DNA template, and PCR grade distilled water. Amplification results obtained through PCR (Kyratec thermocycler) were run on a 1.0% agarose gel prepared in 1 TAE buffer and subjected to electrophoresis at 100 V for 60 min. The gel was then visualised under UV light using an ethidium bromide dye. The size of the amplicons was estimated using 50- and 100-pb DNA size markers. The primer sequences used are presented in Table 2.

## RESULTS

### Identification of the bacterial species

From 246 mussel samples obtained under different selling conditions, 30 *Staphylococcus* isolates were obtained through culture. Through 16S rRNA analysis, it was determined that the 30 bacterial isolates had a high similarity (≥99.12 - 100%) with species registered in the GenBank database. Eight of the isolated species were identified as *Staphylococcus pasteurii*, and 22 were identified as *Staphylococcus warneri*. In this study, Other staphylococcus members were not isolated, except *S. pasteurii* and *S. warneri*.

### Antibiotic resistance findings

The phenotypic and genotypic antibiotic resistance results of the 30 isolates obtained from stuffed mussel samples are presented in Table 3. All *S. warneri* isolates demonstrated resistance to amoxicillin/clavulanic acid and erythromycin among the tested antibiotics. Additionally, seven *S. warneri* isolates exhibited intermediate sensitivity to tetracycline and streptomycin, whereas eight were found to have intermediate sensitivity to tetracycline antibiotics. In *S. warneri* isolates, at least three resistance genes (*BlaTEM*, *tetB-6*, *tetK-8*), and up to eight resistance genes (*BlaTEM*, *tetB-6*, *tetK-8*, *strA-strB*, *aphAI-IAB*, *ermC*) were identified (Table 3).

In this study, it was found that all *S. pasteurii* isolates were resistant to amoxicillin/clavulanic acid. Eight isolates of *S. pasteurii* were determined to have intermediate sensitivity to ampicillin and streptomycin antibiotics. Resistance genes *blaTEM*, *strA-strB*, and *aphAI-IAB* were identified in all *S. pasteurii* isolates.

## DISCUSSION

In the present study, it was determined that antibiotic-resistant *S. warneri* and *S. pasteurii* contaminate stuffed mussel samples. Similarly, In Italy, samples taken from the preparation counters of ready-to-eat meal companies revealed the isolation of *S. pasteurii* and *S. warneri*. These isolates were reported to be resistant to antibiotics at rates of 83.3% and 42.9%, respectively, among *Staphylococcus* species (Marino et al., 2011). In Türkiye, *S. warneri* was isolated from fish gills sold at counters, whereas *S. pasteurii* was isolated from the skin and gills (Çoban & Yaman, 2023).

Similar to the present study, antibiotic resistance of *S. warneri* isolated from seafood was reported in a previous study. Isolates of *S. warneri* from sea fish meat have been reported to be resistant to penicillin, ampicillin, tetracycline, erythromycin, and/or

**Table 2.** List of primers for the detection of antimicrobial resistance genes.

Targeted gene		Sequence (5'-3')	Amplicon (pb)	References
β-lactamases	<i>bla<sub>TEM</sub></i>	F CATTTCGGTGTGCGCCCTATTC	800	Dallenne et al. 2010
		R CGTTCATCCATAGTTGCCTGAC		
	<i>bla<sub>S</sub>Hv</i>	F AGCCGCTTGAGCAAATTAAC	710	
		R ATCCCGCAGATAAATCACCAC		
<i>bla<sub>C</sub>rx-M</i>	F CGCTTTGCGATGTGCAG	550	Paterson et al. 2003	
	R ACCGCGATATCGTTGGT			
	<i>mecA</i>	F GTGAAGATATACCAAGTGATT	150	Alfatemi et al. 2014
		R ATGCGCTATAGATTGAAAGGAT		
Tetracycline	<i>tetA</i>	F GTAATTCTGAGCACTGTCGC	950	Sengeløv et al. 2003
		R CTGCCTGGACAACATTGCTT		
	<i>tetB</i>	F CTCAGTATCCAAGCCTTTG	400	Sunde and Sørnum, 2001
		R CTAAGCACTGTCTCCTGTT		
	<i>tetE</i>	F GTGATGATGGCACTGGTCAT	1100	Sengeløv et al. 2003
		R CTCTGCTGTACATCGCTCTT		
<i>tetK</i>	F TATTTTGGCTTTGTATTCTTTTCAT	1150	Trzcinski et al. 2000	
	R GCTATACCTGTCCCTCTGATAA			
<i>tetM</i>	F ACAGAAAGCTTATTATATAAC	171	Aminov et al. 2001	
	R TGGCGTGTCTATGATGTTAC			
Aminoglycoside resistance	<i>strA-strB</i>	F TATCTGCGATTGGACCCTCTG	540	Sunde and Sørnum 2001
		R CATTGCTCATCATTTGATCGGCT		
	<i>aphAI-IAB</i>	F AAACGTCTTGCTCGAGGC	460	Frana et al. 2001
		R CAAACCGTTATTCATTCTGTA		
<i>aac(3)-IIa</i>	F ATGGGCATCATTGCGACA	750	Dai et al. 2010	
	R TCTCGGCTTGAACGAATTGT			
<i>aac(6')-Ib</i>	F TTGCGATGCTCTATGAGTGGCTA	480	Katalin, 2000	
	R CTCGAATGCCTGGCGTGTTT			
Macrolides	<i>ermA</i>	F GTTCAAGAACAATCAATACAGAG	420	Lina et al. 1999
		R GGATCAGGAAAAGGACATTTTAC		
	<i>ermB</i>	F CCGTTTACGAAATTGGAACAGGTTAAAGGGC	360	
		R GAATCGAGACTTGAGTGTGC		
	<i>ermC</i>	F GCTAATATTGTTAAATCGTCAATTCC	570	
		R GGATCAGGAAAAGGACATTTTAC		

oxacillin, whereas *S. pasteurii* isolates have been reported to be resistant to penicillin, oxacillin, ampicillin, and/or erythromycin antibiotics (Regecová et al., 2014). Moreover, isolates of *S. warneri* from fermented sausage products on the market were reported to be resistant to ampicillin, erythromycin, kanamycin, penicillin G, cefalotin, and ceftiofur antibiotics, whereas *S. pasteurii* was reported to be resistant only to erythromycin (Geniş & Tuncer, 2018). In a study conducted by Çavdar et al. in 2022, *S. warneri* was isolated from hospital water tanks, showers, and taps, whereas *S. pasteurii* was isolated only from the water tank.

*S. warneri* causes disease in rainbow trout in Türkiye and shows resistance to gentamicin, oxacillin, colistin, oxytetracycline, tylosin, spectinomycin, ampicillin, clindamycin, and erythromycin antibiotics (Diler et al., 2023).

To date, there is limited information regarding the presence of antibiotic resistance genes in isolated species of *S. pasteurii* and *S. warneri* from aquatic products. For example, *S. pasteurii* isolat-

ed from Jeotgal has been reported to be resistant to linezolid, penicillin, and trimethoprim antibiotics, with trimethoprim resistance associated with carrying the *dfrA* gene (Jeong and Lee, 2015).

## CONCLUSION

In this study, resistance to amoxicillin/clavulanic acid and erythromycin was observed in all *S. warneri* isolates. In addition, all *S. pasteurii* isolates were found to be resistant to amoxicillin/clavulanic acid. In *S. warneri* isolates, the identified resistance genes include *Bla<sub>TEM</sub>*, *tetB-6*, *tetK-8*, *strA-strB*, *aphAI-IAB*, and *ermC*, whereas in *S. pasteurii* isolates, the identified resistance genes are *bla<sub>TEM</sub>*, *strA-strB*, and *aphAI-IAB*. Consequently, this study will provide a valuable reference for enhancing monitoring strategies in stuffed mussel production. In further studies, the level of public health risk posed by the product should be determined. Therefore, it is necessary to clarify the pathogenic potential and mechanisms of the pathogens identified in stuffed mussels.

**Table 3.** *Staphylococcus* isolates identification results and phenotypic/genotypic antibiotic resistance profiles.

No	Species	Similarity*	Antibiotics	bla <sub>TEM</sub>	tetB-6	Resistance Gene	ermC
1	<i>Staphylococcus warneri</i>	MK737139 (99.78%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	strA-strB	ermC
2	<i>Staphylococcus warneri</i>	MT453899 (99.93%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6		
3	<i>Staphylococcus warneri</i>	MW527395 (100%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
4	<i>Staphylococcus pasteuri</i>	MT539733 (99.92%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
5	<i>Staphylococcus pasteuri</i>	MF429378 (99.72%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
6	<i>Staphylococcus warneri</i>	MT328647 (100%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
7	<i>Staphylococcus pasteuri</i>	OR976013 (99.12%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
8	<i>Staphylococcus warneri</i>	MF681861 (99.14%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	strA-strB	ermC
9	<i>Staphylococcus warneri</i>	KY218866 (99.13%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
10	<i>Staphylococcus warneri</i>	KR809427 (99.14%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	strA-strB	ermC
11	<i>Staphylococcus warneri</i>	ON386170 (99.14%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	strA-strB	ermC
12	<i>Staphylococcus warneri</i>	KT906680 (99.95%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
13	<i>Staphylococcus warneri</i>	MW148447 (99.55%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
14	<i>Staphylococcus warneri</i>	MN512291 (99.15%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
15	<i>Staphylococcus warneri</i>	KX826983 (99.15%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
16	<i>Staphylococcus warneri</i>	MZ043864 (99.14%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
17	<i>Staphylococcus warneri</i>	LN794823 (99.15%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
18	<i>Staphylococcus pasteuri</i>	KJ486553 (99.15%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
19	<i>Staphylococcus warneri</i>	MF681861 (99.20%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	ermC	
20	<i>Staphylococcus pasteuri</i>	KR809418 (99.70%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
21	<i>Staphylococcus warneri</i>	MG800691 (99.20%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	ermC	
22	<i>Staphylococcus warneri</i>	MZ043864 (99.20%)	E, AMC, TE	bla <sub>TEM</sub>	tetB-6	ermC	
23	<i>Staphylococcus warneri</i>	MK737139 (99.15%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
24	<i>Staphylococcus warneri</i>	MW527395 (99.98%)	E, AMC, TE*, S*	bla <sub>TEM</sub>	tetB-6	strA-strB	ermC
25	<i>Staphylococcus warneri</i>	MT328647 (99.82%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
26	<i>Staphylococcus pasteuri</i>	OR517214 (99.73%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
27	<i>Staphylococcus pasteuri</i>	KP261074 (99.56%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	
28	<i>Staphylococcus warneri</i>	KP860608 (99.13%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
29	<i>Staphylococcus warneri</i>	KP261060 (99.13%)	E, AMC, TE*	bla <sub>TEM</sub>	tetB-6	ermC	
30	<i>Staphylococcus pasteuri</i>	KT582293 (99.37%)	AMC, AMP*, S*	bla <sub>TEM</sub>	strA-strB	aphA1-IAB	

\*Intermediate, \*\*Accession numbers and Sequence result (similarity with 16S rRNA), AMC: amoxicillin/clavulanic acid, AMP: ampicillin, E: erythromycin, TE: tetracycline, S: streptomycin

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## Effect of Curry Leaf (*Murraya koenigii*) as Edwardsiellosis Treatment on Gourami Fish (*Osphronemus goramy*)

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### ABSTRACT

The primary objective of this study was to explore the potential of *Murraya koenigii* as a natural alternative to synthetic antimicrobial agents for the treatment of edwardsiellosis in gourami fish (*Osphronemus goramy*). This research employed a combination of both experimental and descriptive methods. The research followed a two-stage approach. In the first stage, antibacterial compounds were extracted and characterized from *M. koenigii* leaves, followed by an assessment of their antibacterial activity against *Edwardsiella tarda*. The second stage involved applying the crude extract of *M. koenigii*, known for its antibacterial properties, to gourami fish infected with *E. tarda*. The findings revealed that *M. koenigii* predominantly contains alkaloids, terpenoids, saponins, flavonoids, and tannins, all of which exhibit antibacterial activity. The application of the crude *M. koenigii* extract had a substantial impact on the hematological profile of the infected gourami fish. The most effective dose in treating Edwardsiellosis in gourami fish was found to be 600 mg/L, as it significantly improved various hematological parameters, including hematocrit, hemoglobin, erythrocyte count, leukocyte count, lymphocytes, monocytes, and neutrophils. Additionally, histopathological analysis showed improvements in tissue conditions, with lower scores for degeneration, congestion, and necrosis. Furthermore, the survival rate of gourami fish reached its highest at 83.33% when treated with the 600 mg/L dose of *M. koenigii* extract. These results suggest the potential of *M. koenigii* as an effective alternative for the treatment of Edwardsiellosis in gourami fish. However, further research is needed to assess the practical application of *M. koenigii* extract in gourami fish culture.

**Keywords:** Gourami Fish, Bacterial Fish Diseases, Edwardsiellosis, Fish Diseases Treatment, *Murraya koenigii*

### INTRODUCTION

Gourami fish (*Osphronemus goramy*) is a freshwater fish species that has been cultured in the South-east Asian region; its maintenance cost is relatively low, and it has adaptability with low dissolved oxygen content. Gourami fish also has a relatively stable high selling price (2.28 USD/kg) (Al-Baiquni, 2019). However, an intensive maintenance strategy to pursue production targets evokes damage to

the immune system and susceptibility to diseases which evokes high economic losses if not resolved (Abdel-Latif et al., 2020).

*Edwardsiella tarda* is a species of the Gram-negative bacteria, which poses a significant challenge to the aquaculture industry. It is responsible for causing edwardsiellosis, a disease that has been identified as a major threat to various economically valuable fish species globally (Li et al., 2019).

This pathogen has led to substantial losses in the aquaculture sector, with significant impacts observed in countries such as the United States of America, Japan, and various European and Asian countries. In scenarios where fish are raised in ponds with limited water circulation, losses due to *E. tarda* infections can be particularly severe, sometimes reaching as high as 50% (Kole et al., 2017).

The effective treatment of edwardsiellosis, caused by *E. tarda* infection, has been achieved through the use of antibiotics such as tetracycline and kanamycin. However, the widespread and inappropriate use of antibiotics has led to significant problems. These challenges encompass the development of antibiotic resistance in *E. tarda* and the potential presence of antibiotic residues in human populations (Xu et al., 2019). As highlighted by Harikrishnan et al. (2020), the use of antibiotics and chemical agents in aquaculture can also pose risks, including concerns related to water toxicity, public health, and environmental damage.

One potential solution to address the aforementioned challenges involves the utilization of a natural bioactive crude extract derived from curry leaves, *Murraya koenigii*, which contains antibacterial compounds. *M. koenigii* is a plant belonging to the Rutaceae family and is commonly found in tropical and subtropical regions. Extensive phytochemical analyses of *M. koenigii* have revealed a wide range of natural compounds, including alkaloids, sesquiterpenes, essential oils, and alkenes (Patil et al., 2024). Different parts of the *M. koenigii* plant exhibit various biological activities, including anti-inflammatory, antioxidative, nephroprotective, hepatoprotective, anti-listerial, and antibacterial properties (Ma et al., 2019). To gain a deeper understanding of the bioactive components of *M. koenigii*, researchers conducted GC-MS (Gas Chromatography-Mass Spectrometry) analysis. They investigated the chemical composition of the ethanol extract of *M. koenigii* using GC-MS and compared the mass spectra of the identified compounds in the extract to the National Institute of Standards and Technology (NIST) library (Azhagu et al., 2021). The analysis of the ethanol extract of *M. koenigii* revealed the presence of two primary compounds: 1-methyl-pyrrolidine-2-carboxylic acid, which constituted 69.00% of the extract, and ethyl  $\alpha$ -glucopyranoside, making up 13.36% of the extract (Salikutty et al., 2012).

Numerous investigations have explored the potential antibacterial properties of *M. koenigii*, but its specific use as an antibacterial agent against *in vitro* antibacterial activity was investigated by Syaifurrisal et al. (2021) and its application in the treatment of fish has never been explored. It is imperative to initiate research and studies focused on the identification of active compounds within *M. koenigii* with antibacterial properties. Furthermore, these studies should investigate the feasibility of employing these compounds as potential drug candidates for the treatment of *E. tarda* infections in gourami fish. This research should encompass comprehensive hematological and histopathological examinations to determine the potential therapeutic effects.

## MATERIAL AND METHODS

### Experimental fish and bacteria

Experimental gourami fish samples (7-10 cm) were provided from fish farms located in Tulungagung, East Java, Indonesia. To con-

duct antibacterial activity testing, a pure culture of *E. tarda* was acquired from BUSKIPM Jakarta. The culture and revitalization of *E. tarda* bacteria were facilitated using TSB (Tryptic Soy Broth) as the growth medium. The LD<sub>50</sub> (Lethal Dosage 50) test was carried out to determine the density and time required for bacteria to kill 50% of the experimental fish (Rattanachaikunsopon & Phumkha-chorn, 2010). The LD<sub>50</sub> test uses a pure culture of *E. tarda* bacteria with a density 10<sup>9</sup> cells/ml, 10<sup>8</sup> cells/ml, 10<sup>7</sup> cells/ml, 10<sup>6</sup> cells/ml, and 10<sup>5</sup> cells/ml. The *E. tarda* used in the study had a density of 2.53 x 10<sup>7</sup> cells/mL, as determined by the LD<sub>50</sub> results. In this test, fish were put into treatment containers with a stocking density of 10 fish/container.

Antibacterial activity testing was carried out using a disc test to examine the inhibitory power of the crude extract of *M. koenigii* leaves on *E. tarda* bacteria. According to Ulmursida et al., (2017) in testing how big the impact of giving the extract is and if the diffusion method shows positive results, the clear zone area results will be obtained by reducing the diameter of the clear zone by the diameter of the paper disc (6 mm). *E. tarda* cell damage was analyzed by comparing SEM photos (normal conditions and after the bacteria were treated with crude extract of *M. koenigii* leaves so that a picture of bacterial cell wall damage was visible. According to Nursidika et al., (2014), determining the antimicrobial mechanism can be done using the SEM method. The working principle of SEM is the creation of images based on the detection of new (secondary) or reflected electrons that appear or emerge from the sample surface when the sample surface is scanned using an electron beam.

### Experimental design

To infect experimental fish with *E. tarda* bacteria, the experimental fish were soaked in a pure culture of *E. tarda*. Soaking was carried out in an aquarium measuring 30 x 30 x 30 cm with a density of 10 fish in 20 liters of water at a temperature of 27-30°C. Observations of fish death were then carried out for 96 hours, according to the statement of Wulandari et al. (2014), since this was the time commonly used in short-scale bioassays. This research employed a combination of both experimental and descriptive methods. The experimental approach was employed to obtain quantitative data for this study, while the descriptive method was utilized to comprehensively depict the natural bioactive components found in *M. koenigii* and their effects on the hematological and histopathological aspects of gourami fish. In this study, various treatments were administered, including negative control (K-) infected fish that were not treated, positive control (K+) normal fish that were not infected, as well as doses of 500 mg/L, 600 mg/L, 650 mg/L, and 700 mg/L (three repetitions, 10 fish each). The selection of these dosages was based on prior *in vitro* research conducted by Syaifurrisal et al. (2021).

### Extraction of *M. koenigii*

The *M. koenigii* leaves utilized in this study were collected from the Ampel Religious Tourism area of Surabaya, Indonesia, and extracted with ethanol as the maceration solvent. To ascertain the presence of active compounds in the extract, several tests were conducted by the methodology outlined by Dewi et al. (2019). These tests encompassed phytochemical screening, UV-Vis (Ultraviolet-Visible) analysis, and FTIR (Fourier-transform in-

frared) spectroscopy. The primary objective of phytochemical screening was to detect the presence of active compounds in the plant extract such as saponins, tannins, flavonoids, alkaloids, and terpenoids, within the crude extract of *M. koenigii* leaves.

The extract was then also tested for LC<sub>50</sub> (Lethal Concentration) to determine the concentration of the extract that could cause 50%, to determine a dose that was safe and did not cause death in experimental fish (Suciati et al., 2012). In this test, fish were put into treatment containers with a stocking density of 10 fish/container. Then the aquarium is filled with extract according to the treatment. The behavior, number of fish that died, and the time were recorded. The test was repeated if fish in the control treatment experienced mortality above 10% (Taufik & Setiadi, 2012).

### Histopathology analysis

Edwardsiellosis can be observed with symptoms such as lesions in muscle tissue, skin, and internal organs, such as kidney tissue (Harikrishnan et al., 2020). Kidney tissue can be an important organ in the study of pathogenic mechanisms and immune responses in fish (Rauta et al., 2012; Bailey et al., 2020). The relationship between the severity of histological changes due to bacterial infection and the immune response can be elucidated by the study of these organs. Some studies report kidney tissue is used as histopathology analysis for edwardsiellosis in fish (Cheng et al., 2020; Kalindamar et al., 2020). To determine the characteristics of kidney tissue, histopathological observations of gourami fish were carried out. The staining method used Haematoxylin Eosin previously fixed with 10% formalin and calculations were used to assess the impact of *M. koenigii* extract on edwardsiellosis, as described by Maftuch et al. (2015). Also, the normal and pathological semi-quantitative approach was used to compare normal tissue samples with tissue that had been affected by the pathogen. This involved determining the extent of staining in stained areas and manually calculating the percentage of affected tissue, following the method outlined by Maftuch et al. (2015). This histopathological analysis was performed with three replications, and for each treatment, five distinct views were examined to ensure a comprehensive assessment.

### Blood analysis

Hematological observations encompassed the assessment of several parameters, including hematocrit value, hemoglobin levels, erythrocyte count, leukocyte count, monocyte count, lymphocyte count, and neutrophil count, following the methodology outlined by Maftuch (2018). These observations were conducted by extracting blood from the lateral line of the gourami fish using a 1 ml syringe, which had been preloaded with 0.5 µL of EDTA as an anticoagulant. The blood cell count was then determined. This test aimed to evaluate the condition of infected gourami fish after the administration of *M. koenigii* extract. The blood analysis was performed with three repetitions and monitored before infection, after infection, and after treatment.

### Survival rate

To assess the effectiveness of *M. koenigii* in treating Edwardsiellosis, the survival rate was calculated with the formula given by Kasnir et al. (2023). The research spanned 7 days for survival rate

assessment, involving three replications for each treatment group and a sample of 10 fish in each replication.

$$SR = \frac{N_t}{N_o} \times 100\%$$

Notes:

SR: Survival Rate (%)

Nt: The quantity of fish at a specific time point t (individual)

No: The initial count of fish at the start of the experiment (individual)

### Data analysis

In this study, data analysis was carried out in a completely randomized design (CRD) using the Statistical Package for the Social Sciences (SPSS) 25.0 for Windows software application.

## RESULTS AND DISCUSSION

### Active compound present in *M. koenigii*

The phytochemical tests conducted on the crude extract of *M. koenigii* leaves are summarized in Table 1. The results of the phytochemical screening indicated that the crude extract derived from *M. koenigii* leaves contained various active compounds, including flavonoids, alkaloids, saponins, terpenoids, and tannins. These outcomes are consistent with the findings reported by Fauziah et al. (2014), who also identified alkaloids, steroids, flavonoids, tannins, and phenolic compounds as the natural bioactive constituents present in the crude extract of *M. Koenigii*.

**Table 1.** Phytochemical Test Results.

Active Compound	Result	Description
<b>Flavonoid</b>	+	Formed pink color
<b>Saponin</b>	+	Formed non-permanent foam
<b>Alkaloid</b>		
- Dragendroff	+	An orange precipitate formed
- Meyer	+	A white precipitate formed
- Buchardat	+	A brown precipitate formed
<b>Tannin</b>	+	A blackish brown color is formed
<b>Terpenoid</b>		
- Steroid	-	Formed a bluish green color
- Triterpenoid	+	A brownish-orange color is formed

To validate the presence of the active constituents identified in the phytochemical test results, further analysis of the crude extract from *M. koenigii* was conducted using both a UV-Vis spectrophotometer (Table 2) and an FTIR (Fourier-transform infrared) spectrophotometer (Table 3). The UV-Vis observations indicated that the dominant compounds in the extract were derived from the alkaloid group (with a peak at 3.893), saponin group (peaking at 3.824), and terpenoid group (peaking at 4.016). These groups exhibited notably high absorbance values. Conversely, flavonoids (with a peak at 0.022) and tannins (peaking at 1.590) displayed comparatively lower absorbance values. According to Hammado and Illing (2013), absorbance values can provide in-

sights into the concentration of bioactive compounds within the extract. A higher absorbance value suggests that the absorption band contains specific compounds with a stronger absorption affinity. Furthermore, the FTIR observations unequivocally confirmed the presence of compounds such as flavonoids, alkaloids, saponins, terpenoids, and tannins.

**Table 2.** UV-Vis peak point data from *M. koenigii* extract.

Long Waveform (nm)	Absorbance	Compound	Literature
662.0	0.022	Flavonoid	(Obaseki et al., 2017)
294.9	1.590	Tanin	(Lestari & Sidik, 2013)
220.0	3.480	Alkaloid	(Hammado & Illing, 2013)
217.0	3.577	Alkaloid	(Hammado & Illing, 2013)
213.0	3.893	Alkaloid	(Hammado & Illing, 2013)
211.0	3.824	Saponin	(Amanati et al., 2017)
205.1	4.016	Terpenoid	(Agustini et al, 2019)
202.1	3.879	Terpenoid	(Agustini et al., 2019)

**Table 3.** FTIR spectrophotometer wavelength absorption data from *M. koenigii* extract.

FTIR wavelength (cm <sup>-1</sup> )	Functional groups	Group
1453.868	N-H	Alkaloid (Hammado & Illing, 2013)
1627.321	C=O	Alkaloid (Aksara et al, 2013)
1453.868	C-H alifatik	Alkaloid (Hammado & Illing, 2013)
1163.814	N-H bending	Alkaloid (Aksara et al, 2013)
1069.868	N-H bending	Alkaloid (Aksara et al, 2013)
928.175	Tekuk C- H	Alkaloid (Santi, 2010)
2924.574	-OH	Terpenoid (Agustini et al., 2019)
2855.169	C-H alifatik	Terpenoid (Agustini et al., 2019)
1713.556	C=O	Terpenoid (Agustini et al., 2019)
1314.393	C-O-H	Tanin (Sari et al., 2015)
679.341	C-H bending	Tanin (Sari et al., 2015)
583.419	C-H bending	Tanin (Sari et al., 2015)
1209.989	C-O	Saponin (Bintoro et al, 2017)
835.125	C-H	Flavonoid (Ningrum et al, 2017)

### Histopathology analysis

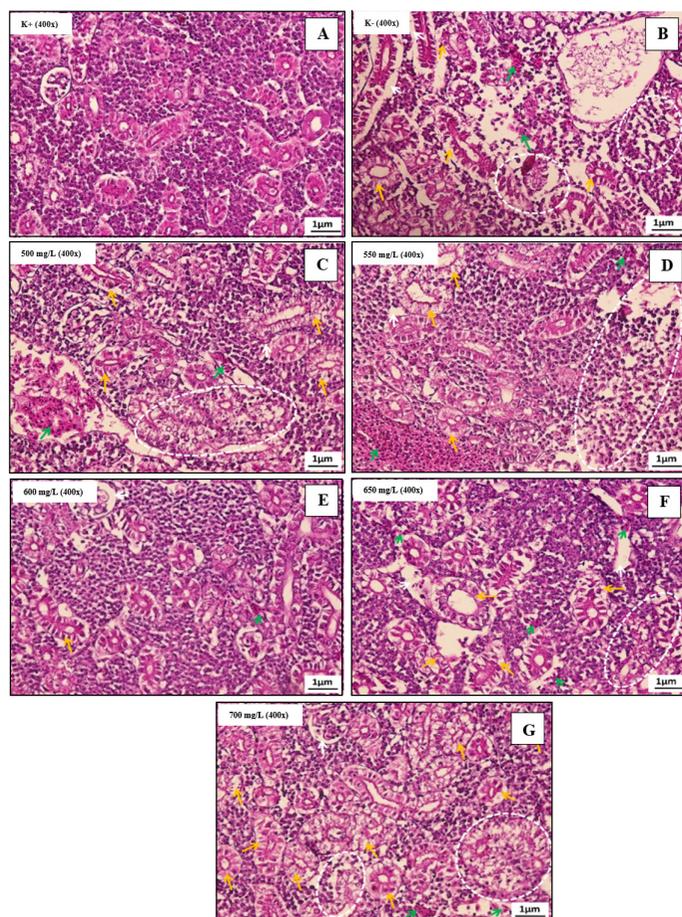
The histopathological images presented in Figure 1 offer valuable insights. The kidney tissue of healthy fish appears normal, showing no signs of cellular damage. The cross-section of the

network, including the glomerulus and tubular tissue exhibits a healthy state. The distal tubule tissue and hematopoietic tissue also display normal conditions, devoid of any damage. This contrasts with infected fish, where congestion is apparent. The reddish color observed in kidney cells is indicative of congestion resulting from an increased presence of blood clots within the blood vessels, as explained by Parameswari et al. (2013). In severe cases, congestion can lead to ruptured blood vessels, ultimately causing cell death or necrosis, characterized by changes in cell nuclei, where the nuclei become denser and darker (a condition known as pyknosis) (Abdelfadeel et al., 2023). Coagulative necrosis, described by Adinata et al. (2012), involves densely packed and fixed protoplasmic cells that can still be observed under a microscope.

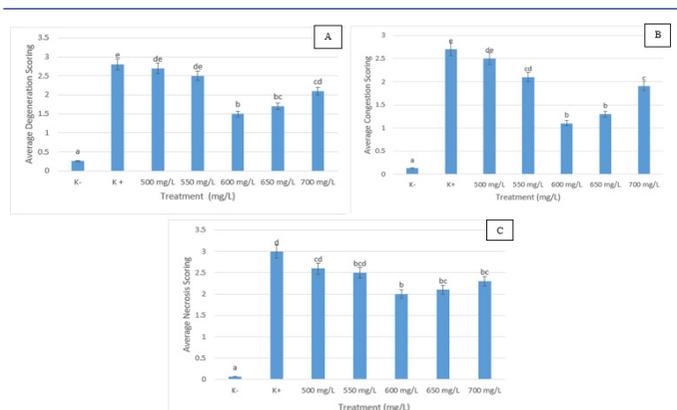
Takashima and Hibiya (1995) define necrosis as a state of reduced tissue activity marked by the progressive loss of cell components, accompanying cell degeneration in organisms. In histopathological terms, the use of *M. koenigii* is shown to inhibit *E. tarda* infection in gourami fish. Many prior studies have explored the application of *M. koenigii* in treating pathogenic bacteria such as *Escherichia coli*, *Aeromonas hydrophila*, *Citrobacter freundii*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus anginosus* in fish *in vitro* (Heny et al., 2011; Najiah et al., 2011). However, this research is the first to demonstrate the effectiveness of *M. koenigii* in treating *E. tarda* infection in gourami fish. Notably, kidney necrosis was observed to increase in average scores at the 650 mg/L and 700 mg/L treatments, indicating that the extract reached its optimal efficacy for treatment following *E. tarda* infection. Doses exceeding this optimal point appeared to result in more severe kidney tissue damage.

According to Kumar et al. (2012), proximal tubular epithelial cells are highly susceptible to the effects of toxic substances. This vulnerability arises because toxic substances tend to accumulate at higher levels in the proximal tubules due to active processes of absorption and secretion in this region. Additionally, the proximal tubules have elevated levels of cytochrome P450 enzymes, which play a role in either detoxifying or activating toxic substances (Ge et al., 2022). Consequently, these tubules are often the primary targets of the adverse effects caused by toxic substances. Continuous exposure of the proximal convoluted tubule to toxic substances can result in cellular injury, ultimately leading to cell death, known as necrosis (Moore et al., 2021).

In the overall evaluation of the comparison to the positive control group, the treatment with *M. koenigii* extract appeared to have a positive impact on mitigating the extent of kidney necrosis (Figure 2). This positive effect can be attributed to the bioactive compounds present in *M. koenigii*'s crude extract, such as alkaloids and flavonoids, which can stimulate the fish's immune defense system. Previous studies have indicated that alkaloids and flavonoids possess antibacterial properties (Donadio et al., 2021; Yan et al., 2021). Alkaloids can disrupt bacterial cell membranes, inhibiting bacterial growth (Jubair et al., 2021), while flavonoids can cause physical membrane disruption and act as inhibitors of ATP synthase (Donadio et al., 2021).



**Figure 1.** Kidney Histopathological Overview of Gourami Fish / 400x. (A) Normal (non-infected) /K+, (B) Infected fish (not-treated) /K-, (C) treated with 500 mg/L. (D) treated with 550 mg/L. (E) treated with 600 mg/L. (F) treated with 650 mg/L. (G) treated with 700 mg/L, (Yellow Arrow: Degeneration; Green Arrow: Congestion; White Circle: Necrotic Area)



**Figure 2.** Histopathological Scoring Test Results (A) Degeneration, (B) Congestion, and (C) Kidney Necrosis of Gourami Fish.

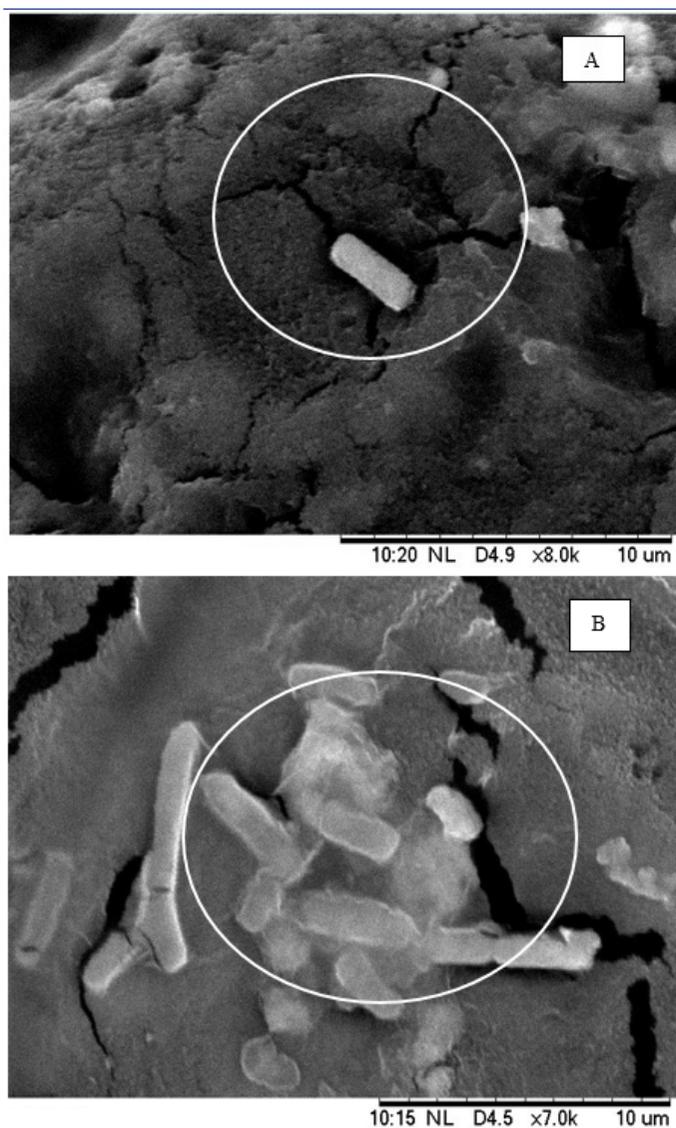
The treatment with the lowest average score for abnormal histopathological findings was observed at a dose of 600 mg/L, which resulted in 1.5 for degeneration, 1.1 for congestion, and 2 for necrosis. This indicates that 600 mg/L was the most effective dosage of *M. koenigii* for treating edwardsiellosis in gourami fish. According to Kim & Yang (2018), employing natural active ingredients at the appropriate dosage can stimulate the immune system and enhance protection against infections. Interestingly, after the decrease in damage scores observed in the extract concentrations ranging from 500 mg/L to 600 mg/L, there was a noticeable increase in congestion at the 650 mg/L and 700 mg/L treatment levels. This suggests that the extract's effectiveness had reached its peak at the optimal dosage, and doses higher than this optimum could become toxic, causing more severe kidney tissue damage. At high doses, the secondary metabolites present in the crude extract of *M. koenigii* leaves can be toxic to gourami fish. According to Rand et al. (2015), as the dose of the extract administered increases, the damage to tissue cells also intensifies. This study also showed that no test animals succumbed during the treatment process because the density of bacteria and the concentration of the extract dose used had been through acute LD<sub>50</sub> and LC<sub>50</sub> toxicity tests to assess the acute safety of a drug or substance to be used.

### SEM test (Scanning Electron Microscopy)

The SEM results offer insights into the effects of the treatment on the structural changes in bacterial cells (Figure 3). Figure 3A shows the morphology of *E. tarda* bacteria with no apparent damage to their cell walls, while Figure 3B presents morphological images of *E. tarda* bacteria that have experienced lysis due to instability in their cell walls. For bacterial fish pathogens, after entering the host body, the first step for inducing an infection is the attachment, colonization, and biofilm formation of the micro-organism on the host cells and tissues which is generally linked to their cell wall proteins. According to Hussain et al (1997), accumulative growth on polymer surfaces resulting in biofilm formation; it was contributing to pathogenicity by reducing the efficacy of host defenses and antimicrobial killing. This instability disrupts bacterial metabolism, depletes ATP reserves, and decreases cell acidity. Several studies have elucidated this phenomenon, which occurs when antibacterial compounds induce changes or lysis in the protein structure, cell wall stability, and bacterial plasma membrane. This is a result of antibacterial compounds (bacteriostatic effect) binding to proteins through hydrogen bonds (Zhou et al., 2022). Alkaloid compounds, known for their antibacterial properties, can interfere with the formation of cross-bridges within the peptidoglycan constituent components of bacterial cells. This interference prevents the complete formation of the cell wall layer, ultimately leading to the removal of the bacterial cells from the host's cell surface as explained by Ernawati and Sari (2015). Antimicrobials are bacteriostatic if they only inhibit bacterial growth as compound application continues. However, if it is stopped or finished, bacterial growth will increase again (Soelama et al., 2015).

### Hematology

The collected data included measurements of hematocrit, hemoglobin, and erythrocyte levels, as depicted in Figure 4. The



**Figure 3.** Morphology of *E. tarda* bacteria after SEM test (Lysis) (A) Without treatment, (B) With treatment of *M. koenigii*.

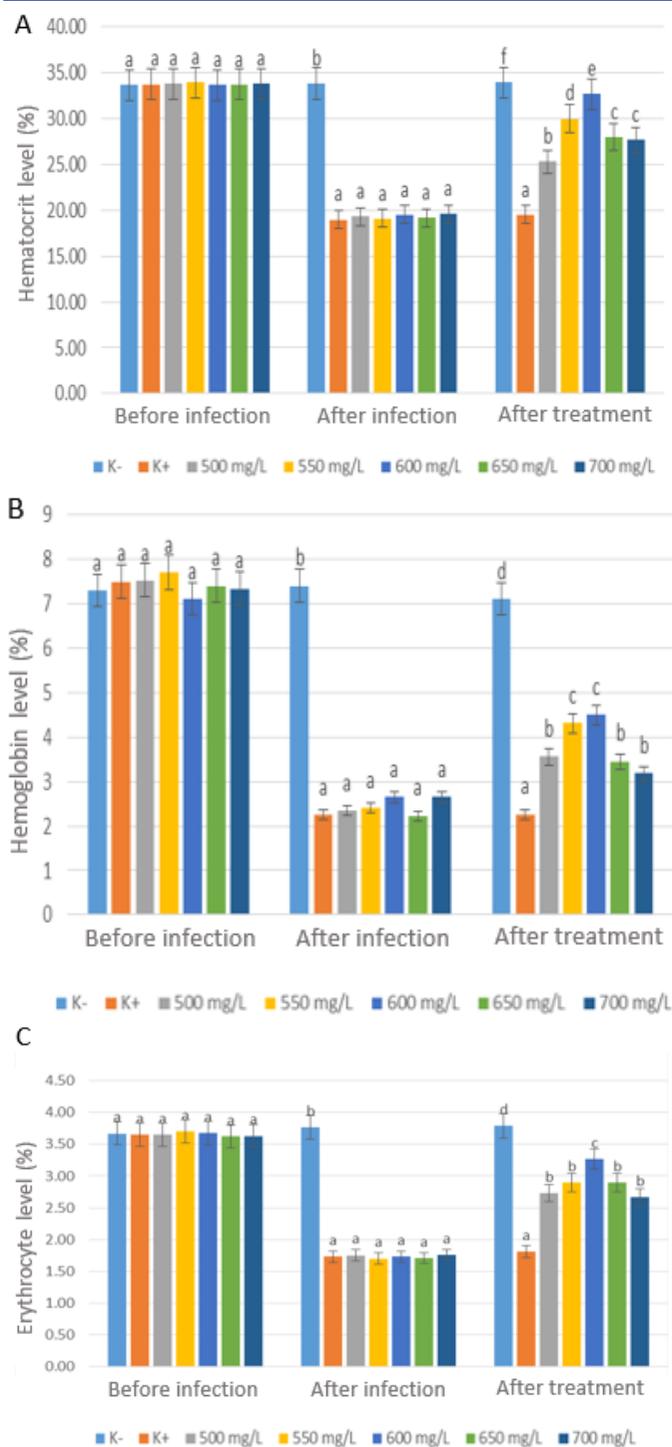
optimal hematocrit value was achieved in the treatment with a dose of 600 mg/L, reaching 32.67%. Regression analysis revealed that the crude extract of *M. koenigii* had a 73% positive effect on improving hematocrit values, which were notably lower after infection, at 19.50%. Similarly, the hemoglobin level showed a significant increase in the 600 mg/L treatment, reaching 4.504 g%. *M. koenigii* demonstrated a 78% effect in improving hemoglobin values, which had declined to 2.652 g% after infection. The highest increase in erythrocyte values was also observed in the treatment with an extract concentration of 600 mg/L, reaching  $3.27 \times 10^6$  cells/mm<sup>3</sup>. The crude extract of *M. koenigii* exhibited a remarkable 78% effect in enhancing erythrocyte values, which had dropped to  $1.73 \times 10^6$  cells/mm<sup>3</sup> after infection. These findings indicate that the extract concentration of 600 mg/L was the most effective in increasing blood parameters after *E. tarda* infection in gourami fish. The increase in hematocrit, hemoglobin, and erythrocyte levels in the blood of

gourami fish can be attributed to the bioactive properties of the extract, such as terpenoids. According to Sundaryono (2011), terpenoids can stimulate erythropoiesis, the process of erythrocyte formation in the bone marrow. The results of UV-Vis observations in this study showed that the dominant compounds in the extract came from the alkaloid group (with a peak of 3.893), the saponin group (peak of 3.824), and the terpenoid group (peak of 4.016). These groups showed very high absorption values.

According to Anderson and Siwick (1993), when fish are infected, their hematocrit levels tend to decrease. A hematocrit value below 20% indicates erythrocyte deficiency. Zorriezahra et al. (2010) stated that a low erythrocyte count and hematocrit level are indicative of anemia in fish. Hardi et al. (2011) also explained that an increase in total erythrocytes in fish blood indicates a homeostatic response in the fish's body. This response aims to produce more blood cells to replace erythrocytes that may have undergone lysis due to infection. Conversely, the decline in hematocrit, hemoglobin, and erythrocyte values at doses of 650 mg/L and 700 mg/L was attributed to excessive dosages causing stress in the fish. This stress was evident from the lower survival rates of the test fish at these two higher doses compared to the 600 mg dose. According to Awad and Awaad (2017), the more extracts used will not necessarily help treatment efforts due to bacterial infections. The active components contained in an extract are toxic to test animals if the concentration is too high. Using natural ingredients at the right dosage can stimulate the immune system and increase protection against infection.

Treatment with various doses of *M. koenigii* also caused different effects on the leukocyte, lymphocyte, monocyte, and neutrophil values of the fish samples (Figure 5). Following treatment with *M. koenigii*, there was a notable decrease in leukocyte values. The most effective dose observed during the study was 600 mg/L, which reduced leukocyte count from  $15.7 \times 10^4$  cells/mm<sup>3</sup> to  $9.6 \times 10^4$  cells/mm<sup>3</sup>. Regression analysis revealed that the 600 mg/L dose of *M. koenigii* had a 61.59% positive effect on improving leukocyte values. This reduction in leukocyte count indicated that the *M. koenigii* extract played a role in the process of treating gourami fish after they were infected with *E. tarda* bacteria. According to Rieger & Barreda (2011), an increase in the number of leukocytes in the blood is an indication of leukocytes mounting an immune response to bacteria, while a decrease in leukocyte count signifies healing and the cessation of the inflammatory process.

Lymphocyte values showed a significant decrease to 48% after the fish were infected with bacteria. However, after treatment, lymphocyte values began to increase, with the most substantial increase occurring at an extract concentration of 600 mg/L, reaching a 68.88% increase. This rise in lymphocyte count following treatment indicated that the compounds present in the crude extract of *M. koenigii* could enhance the gourami fish's immune system in combating *E. tarda* bacterial infection. As stated by Kim & Yang (2018), natural ingredients, when used at the right dosage, can stimulate the immune system and enhance protection against infections.



**Figure 4.** The value of hematocrit (A), hemoglobin (B), and erythrocytes (C) from gourami fish in this study. (K+) Normal (non-infected), (K-) Infected fish (not-treated).

Monocyte and neutrophil values showed a decrease after treatment with *M. koenigii* extract. The best reduction in monocyte values occurred at a concentration of 600 mg/L from 15% to 11.3%. Meanwhile, the highest neutrophil reduction activity occurred at a treatment concentration of 600 mg/L from 17% to

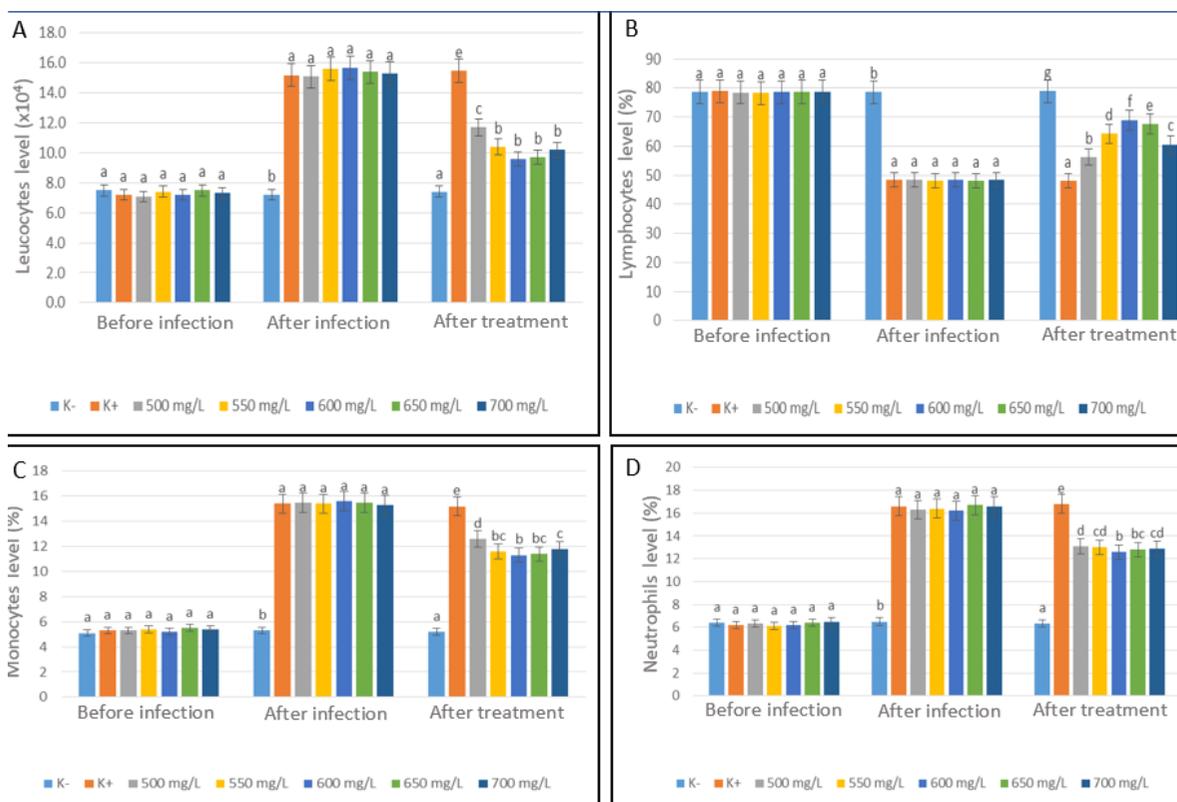
12.6%. The crude extract of *M. koenigii* leaves exhibited the capability to inhibit the growth of *E. tarda* bacteria, leading to a decline in the number of monocytes. This is consistent with Fujaya (2004), suggesting that monocytes leave the bloodstream and migrate to infected areas, where they phagocytize bacteria due to their superior phagocytic capabilities compared to neutrophils. Monocytes serve as macrophages, engaging in the immune system's function of engulfing and destroying pathogenic cells, microorganisms, and foreign substances.

At doses of 650 mg/L and 700 mg/L, the improvement in leukocyte, lymphocyte, monocyte, and neutrophil values was not optimal. This aligns with previous research findings, such as those by Awad & Awaad (2017), which suggest that increasing extract doses may not necessarily enhance treatment efficacy against bacterial infections. Active ingredients within an extract can inhibit pathogen replication and stimulate the innate immune system's defense mechanisms. According to Venkatalakshmi et al. (2016), active compounds like flavonoids can stimulate the release of adrenocorticotropic hormone (ACTH), which, in turn, triggers the adrenal glands to produce cortisol. This cortisol acts as an immunosuppressant. Batool et al. (2020) stated that curry leaf extract exhibited effective immunomodulation in experimental animals through antioxidant and immunosuppressant mechanisms, which are crucial in medicine for reducing an excessive immune response that can occur during infection.

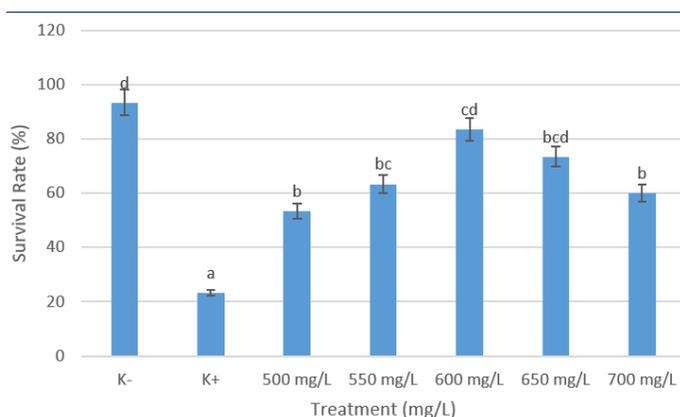
#### Survival Rate

After the administration of *M. koenigii* extracts, the survival rate values were monitored in the experimental groups, and different values were achieved (Figure 6). The highest mean survival rate, at 83.33%, was observed in the 600 mg/L treatment group. This survival rate value closely approached that of the negative control group (not treated), which recorded a survival rate of 93.33%. According to Mulia (2012), providing a booster like an herbal extract can trigger an increase in antibody production because the test fish already possess immune memory, allowing the booster to generate a more robust immune response.

Conversely, the lowest mean survival rate was observed in the positive control treatment group (received infectious agent only and not treated), which recorded a survival rate of 23.33%. The decline in gourami fish survival rates in the 650 mg/L and 700 mg/L treatment groups indicated that the dosage administered had reached its maximum point. Setyani et al. (2018) explained that high mortality occurs when there is a substantial amount of organ damage resulting from bacterial infection and when the extract dosage is sufficient to have a severe impact on the organism. There was increased degeneration, congestion, and necrosis at treatment levels of 650 mg/L and 700 mg/L. This shows that the effectiveness of the extract has reached its peak at the optimal dose, and doses higher than the optimal dose can be toxic, causing more severe kidney tissue damage. At high doses, secondary metabolites contained in the crude extract of *M. koenigii* leaves can be toxic to gourami fish. Rand et al. (2015) explained that as the dose of extract given increases, tissue cell damage also increases.



**Figure 5.** The value of leukocytes (A), lymphocytes (B), monocytes (C), and neutrophils (D) from gourami fish in this study. (K+) Normal (non-infected), (K-) Infected fish (not-treated).



**Figure 6.** Survival Rate of Gourami Fish After Treatment.

## CONCLUSION

In summary, the result of this study revealed that *M. koenigii* leaves are rich in alkaloids, terpenoids, saponins, flavonoids, and tannins so it provides an opportunity for usage as a natural product for the treatment of bacterial infections in fishes. The most effective dose for combating edwardsiellosis in gourami fish is 600 mg/L. This dose significantly influenced various hematological parameters, including hematocrit, hemoglobin, erythrocytes, leukocytes, lymphocytes, monocytes, and neutrophils. Additionally, it had a notable impact on histopathological aspects, with scores indicating mild degeneration, congestion, and necrosis.

To further advance the application of *M. koenigii* extract, additional research and experimentation should be conducted directly within aquaculture activities. This would help to better understand its potential benefits and feasibility in practical aquaculture settings.

**Conflict of Interest:** The authors declare no conflict of interest.

**Ethics Committee Approval:** This research does not harm the experimental animals used.

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# An Overview of Gillnet and Trammel Net Size Selectivity in the Turkish Inland Fisheries

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## ABSTRACT

This study compiled and remodelled length selectivity studies carried out on fish caught with gill nets and trammel nets in the inland fishery in Türkiye and evaluated them based on both the initial reproduction length and minimum landing size of species. The required data for the study were obtained through a literature review. 34 selectivity studies in total were identified, and 26 (76.5%) of them were carried out with gill net while the remaining 8 (23.5%) were conducted with trammel net. 24 of the studies were carried out according to *SELECT* (Share Each Length-class's Catch Total) and 10 of them according to Holt (1963). In conclusion, it was found that minimum conservation reference size (*MCRS*) values determined by the Ministry in the inland fishery in Türkiye were substantially greater than lengths at first maturity (*LFMs*) of species and that fishing gears used for fishing typically tended to fish mature individuals above *MCRS*. The major problem in terms of management is that different ideal mesh sizes are applied to different fish species that are in the same fishing place. Set nets with an ideal-mesh size for a species has a potential of catching individuals below *LFM* of other species in the same fishing grounds. It is thought that this problem can only be resolved with the Ecosystem Approach to Fisheries Management (*EAFm*).

**Keywords:** Inland fisheries, Length at first maturity, Minimum conservation reference size, Small scale fisheries, Sustainable fishery

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## INTRODUCTION

Inland fisheries are defined as "any activity conducted to extract fish and other aquatic organisms from inland waters" by FAO (1997), which has vital importance for especially low-income developing countries in the world, as it supports the livelihood of 60 million people and is a source of food for hundreds of millions more (Moutopoulos et al., 2022; Smith et al., 2005; World-Bank, 2012). In addition to nutritional value, inland fisheries are important for poverty alleviation, gender empowerment, cultural services, ecosystem function and biodiversity (Funge-Smith and Bennett, 2019). Inland fisheries provide employment to millions of people in the world (Rabuffetti et al., 2022). The global capture fisheries production was 90.3 million tonnes in 2020, 12.7% of

which was obtained from inland fisheries (FAO, 2022). The ratio of inland based capture fisheries production of Türkiye is almost 9.1% of the total production (Anonymous, 2021).

In general, inland fisheries are undervalued and ignored as a national or regional priority, despite their importance, however most sustainably generated source of animal protein of the world may be well-managed inland fisheries (Cooke et al., 2016; Moutopoulos et al., 2022). Marine-based fisheries are always very popular among managers and scientists due to their economic importance; however, inland fisheries are also quite important as marine fisheries in terms of considerable livelihood impacts in some countries (such as Bangladesh, Myanmar, Uganda, Nigeria, Cambodia, etc.)



Lakes, rivers, streams, canals, reservoirs, and other land-locked waters are defined as inland waters (FAO, 2014; Lynch et al., 2016). Commercial inland fishing occurs on mainly lakes and dam lakes. Fisheries cooperatives or private entrepreneur have to take a licence from Ministry of Forestry and Agriculture and should be pay a hire charge to the government. A total of 87 dam lakes, 16 natural lakes and three rivers were licenced by the ministry for fish production to inland fishermen in 2022 (fisheries cooperatives hired 82 fishing areas, while private entrepreneurs hired 24) (Anonymous, 2022a). Although Türkiye has 320 natural lakes (Anonymous, 2022b), the majority of them dry up in the summer, making them unsuitable for fisheries. The most important inland fishing areas of Türkiye are Van Lake (eastern Anatolia, 3, 713 km<sup>2</sup>), Beyşehir Lake (southwestern Anatolia 656 km<sup>2</sup>), Eğirdir Lake (south-western Anatolia 482 km<sup>2</sup>), Atatürk Dam Lake (south-eastern Anatolia, 817 km<sup>2</sup>), Keban Dam Lake (eastern Anatolia, 675 km<sup>2</sup>) and Ilisu Dam Lake (south-eastern Anatolia, 313 km<sup>2</sup>).

Inland fisheries are carried out in a small-scale (*SSFs*) concept in Türkiye. Using the efficient fishing gears, such as purse seine and trawl are banned in inland fisheries by legislation. The Turkish inland fisheries fleet consists of 3181 vessels, 97.36% of them are less than 10 m (Anonymous, 2021). Gillnets (tarek, common carp, gibel carp, pikeperch), trammel nets (common carp, gibel carp, European catfish), fyke nets (crayfish, European ell, northern pike), long lines (northern pike, pikeperch) and beach seines (sand smelt) are used by fishermen for catching the aquatic species. Most (94.0%) of the species caught from Türkiye's inland waters are fish, while others are crayfish, crabs, frogs, and snails (Turkstat, 2022).

Capture based inland fisheries production has decreased in Türkiye year by year; the total production declined 22.75% from 2000 (42,824 tonnes) to 2020 (33,119 tonnes). Increased fishing pressure, damming of rivers, deforestation, water pollution, and inadequate practices for managing fisheries are serious challenges to the sustainability of the inland waters (Barletta et al., 2010; Rabuffetti et al., 2022); however, managing the harvest of freshwater species is important for both food security and reducing biodiversity loss (Shephard et al., 2022; Tickner et al., 2020)

It is very important to use selective fishing gears for the sustainability of the natural fish stock in addition to determining the maximum sustainable yield (*MSY*) or the total allowable catch (*TAC*). Removing non-target species and under-sized fish that have not yet reproduced from the catch composition can only be achieved using the high size and species-selective fishing gear.

Gill nets (*GNs*) and trammel nets (*TNs*) which are widely used especially in developing countries in small-scale fishery because they are productive and relatively cheap (Acosta and Appeldoorn, 1995) are widely used by fishermen in the inland fishery in Türkiye. Set nets (*GNs* and *TNs*) rigged with monofilament material are typically preferred by fishermen instead of those rigged with multifilament material because they are (i) relatively cheap, (ii) long-lasting, (iii) resistant to contamination, (iv) easy to carry due to their lightness, (v) not gaining weight by taking in water when they are in the water.

In gilling type fishing, small fish may escape from the gill nets as they pass through the mesh and big fish may do the same as their heads do not fit into the mesh (Pope et al., 1975), in this case, gill nests can only catch a range of length where mesh size is larger than the fish head but smaller than fish body (Yüksel and Aydın, 2012). If a suitable mesh size is determined, both fish which are below the initial reproduction length and which have high reproduction potential (super-spawners) will be concurrently protected (Cilbiz et al., 2022). For this reason, set net selectivity parameters are extremely important scientific data for sustainable management of the natural fish stock. Both the FAO and the European Commission encourage the use of more selective fishing practices to reduce or eliminate bycatch and improve sustainability (Pérez Roda et al., 2019; Suárez et al., 2021).

Despite being highly selective, set nets to be used for species having different minimum landing sizes (*MLS*) (or in other words length at first maturity) are likely to capture bycatch. Therefore, knowing about the interaction between the species fished, minimum landing sizes and mesh sizes will significantly contribute to the maintenance of stocks. In this context, this study compiled and remodelled size selectivity studies conducted on the commercial fish species in the inland fishery in Türkiye and evaluated based on both the initial reproduction size and minimum landing size of species.

## MATERIAL AND METHODS

### Review of set net fishing technology and selectivity properties

Certain technical characteristics of *GNs* and *TNs*, and data needed for setting forth the model used for determining selectivity and the results achieved were obtained through literature review. To this end, peer-reviewed scientific journals and grey literature (e.g. conference proceedings, scientific research project final reports) that published the results of studies performed in the inland fishery in Türkiye were searched. In this section, (i) manufacturing technique for set net (gill net or trammel net), (ii) mesh size of nets tested (*MS*: stretched mesh size in mm), (iii) estimated model length for respective *MS* (*ML*: in cm) and (iv) methods used for modelling selectivity were investigated.

### Review of biological - administrative reference points and commercial fish species

Conformity of estimated model lengths with the biological characteristics of species needs to be set forth in order to reveal the contribution of the results of selectivity studies to the sustainability of stocks. In this context, a literature review was made to (i) determine the commercial species in Türkiye, (ii) for which of the commercial species *GNs* and *TNs* selectivity studies are carried out, (iii) present lengths at first maturity (*LFM*) and (iv) minimum conservation reference sizes (*MCRS*) for those species. In case of presence of multiple studies on the same species, the most recent study was taken into account. Data needed were taken from peer-reviewed scientific journals, governmental institutions' data and legislation review.

### Statistical approach

The relationship between *MS* and *ML* values obtained in selectivity studies was explained using simple linear regression model in the study. The model is described as follows;

$$y = b \cdot x + \varepsilon$$

where:

y: denotes the response variable (MS)

x: denotes the predictor variable (ML)

b: the regression coefficient

$\varepsilon$ : is the measurement error and any variation unexplained by linear model

For species with only one study, linear regression parameters were taken as basis, however, data were bootstrapped in case of presence of two or more studies. A simple linear regression based bootstrapping resampling method was used for estimating the uncertainties for reported selectivity data. 1000 times bootstraps were applied on MS~ML data by using tidymodels (v1.0.0) (Kuhn and Wickham, 2020) in RStudio software.

The ideal MS were computed by using regression model parameters for reference point LFM and MCRS separately. In case of existence of a legal length limit, LFM was primarily taken into account; if there was no available LFM value determined for a species, then MCRS was taken as the valid biological reference point.

## RESULTS

There are almost 20 different fish species caught in Türkiye's inland fisheries (excepting the lagoon areas and other aquatic products such as crustaceans) by the 2021 data, and total production was 30,309 tonnes (Turkstat, 2022). It is determined that selectivity studies were conducted for 14 different fish species *Arabibarbus grypus*, *Alburnus tarichi*, *Blicca bjoerkna*, *Capoeta antalyensis*, *Cyprinus carpio*, *Carassius gibelio*, *Capoeta umbla*, *Esox lucius*, *Luciobarbus esocinus*, *Rutilus rutilus*, *Oncorhynchus mykiss*, *Scardinius erythrophthalmus*, *Sander lucioperca*, and *Vimba vimba*). In this context, it can be said that the set net selectivity is known for 70.0 % of caught fish species.

As a result of the literature review, 34 set net selectivity studies were identified in total. 26 (76.5%) of these studies were carried out with GNs and the remaining 8 (23.5%) were carried out with TNs (Table 1). 24 of the studies were conducted according to SELECT (Share Each Length-class's Catch Total) and 10 of them were conducted according to Holt (1963). As for the number of studies per species; 10 studies were carried out for *C. carpio*, seven for *C. gibelio*, four for *S. lucioperca*, two for *E. lucius*, two for *A. tarichi* and one study for each of all other species. When looked at the methods used for estimating selectivity, it was seen that SELECT (Share Each Length-class's Catch Total) method was widely preferred (70.6%) and Holt (1963) method was partly (29.4%) preferred (Table 1). Minimum conservation reference sizes (MCRS) determined for species by the Ministry of Agriculture and Forestry were found to vary between a total length range of 18 to 50 cm and no MCRS was determined for six species (*B. bjoerkna*, *C. gibelio*, *R. rutilus*, *O. mykiss*, *S. erythrophthalmus* and *V. vimba*) (Anonymous, 2020). Length at first maturity values were known for a major portion (76.9%) of species for which a set net length selectivity study was performed whereas only for three

species (*A. grypus*, *B. bjoerkna* and *C. antalyensis*) LFM values were not known. MS of GNs used in selectivity study varied between 16-200 mm while MS of TNs varied between 34-160 mm (Table 1). Despite being the second mostly fished species in inland fishery in Türkiye, no MCRS was determined for *C. gibelio*. In this study, 25 cm of minimum economic length arising under market conditions was considered MCRS. For species for which more than one LFM reporting was provided for the same species and for species for which separate LFM values were reported for female-male individuals in the same study, the lower value was preferred as the reference LFM value with a protectionist approach. For example, out of LFM values reported by Yüce et al. (2016) as 30.85 for male individuals and as 32.01 for female individuals of *C. carpio*, the lower value proposed for male individuals was taken as reference.

The ideal mesh size for fishing the relevant species was determined in line with the results of the selectivity study (Table 2). In calculations carried out based on MS~ML relationship, MCRS value was primarily taken as basis as the point of intersection, and LFM value was used in the absence of MCRS.

As there were adequate number of studies for the species of *C. carpio* (GNs-TNs), *C. gibelio* (GNs-TNs), *E. lucius* (GNs), and *S. lucioperca* (GNs), ideal mesh sizes were determined according to bootstrap analysis (Figure 1). Ideal mesh sizes of other species given in Table two were determined according to simple linear regression model. Although there were 2 studies for *A. tarichi*, the fact that only two panels were used in the study carried out by Çetinkaya et al. (1995) made bootstrapping of data impossible. For this reason, the ideal mesh size for the species *A. tarichi* was given according to simple linear regression model. The ideal mesh size could not be modelled for *B. bjoerkna* which has neither an MCRS value nor an LFM value although a selectivity study was conducted on it. It was seen that MCRS values declared for all other species except for *C. umbla* were higher than (*C. carpio*, *C. gibelio*, *E. lucius*, *S. lucioperca*, *A. tarichi*) or equal to (*A. grypus*, *L. esocinus*) LFM values.

When MS~ML relationship graphs given in Figure 1 are reviewed, it is seen that confidence interval for TNs is wider compared to that of GNs considering the studies carried out on the same species. Mesh sizes of the nets used for *E. lucius* ve *S. lucioperca* selectivity are smaller than those of the nets used for *C. carpio* ve *C. gibelio* (Figure 1).

In order to observe which net mesh size corresponds to which reference point of which species; model lengths of set nets according to species and reference points (MCRS - LFM) are given as a whole in Figure 2. A green shaded area remaining on the right of the orange region for the same species means a greater MCRS than LFM for the species in question (i.e. MCRS/LFM is greater than 1). Disappearance of green shaded area may result either from the fact that LFM of the species is not known or MCRS limit is not available in commercial fishing (e.g. *R. rutilus*, *S. erythrophthalmus*, *V. vimba*). The most notable point in Figure 2 is that modelled mesh size which is in the confident area for a species corresponds to the unconfident area for another. For example, mesh size of 52.9 mm which is considered ideal for *S. lu-*

**Table 1.** Summary of the selectivity studies and the parameters considered for the analysis.

Gear	Species	MCRS (cm)	LFM (cm)	MS (mm)	ML (cm)	Sel EM	Reference
GNs	<i>Arabibarbus grypus</i>	45	-	40-80	40.12-80.24	Sel	(Anonymous, 2020a; Yuksel et al., 2020)
TNs	<i>Alburnus tarichi</i>	18	13.68	34-44	15.7-20.3	Holt	(Anonymous, 2020a; Çetinkaya et al., 1995; Elp, 1996)
GNS	<i>Alburnus tarichi</i>	18	13.68	16-48	8.5-25.4	Sel	(Anonymous, 2020a; Demirool and Cilbiz, 2023; Elp, 1996)
GNs	<i>Blicca bjoerkna</i>	-	-	40-52	9.8-13.8	Holt	(Balık and Çubuk, 2001)
GNs	<i>Capoeta antalyensis</i>	20	-	32-90	14.8-41.7	Sel	(Cilbiz and Yalim, 2017)
GNs	<i>Cyprinus carpio</i>	40	30.85	40-140	12.4-43.4	Sel	(Anonymous, 2020a; Aydın et al., 2016; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	130-160	53.3-65.6	Sel	(Anonymous, 2020a; Şen, 2016; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	140-200	56.9-84.2	Sel	(Anonymous, 2020a; Dereli et al., 2022; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	70-140	18.1-42.4	Holt	(Anonymous, 2020a; Balık, 1999a; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	56-90	17.6-27.5	Holt	(Anonymous, 2020a; Özyurt and Avşar, 2005; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	90-120	30.0-43.4	Holt	(Anonymous, 2020a; Yağcın, 2006; Yüce et al., 2016)
GNs	<i>Cyprinus carpio</i>	40	30.85	90-120	30.0-37.1	Holt	(Anonymous, 2020a; Dartay and Ateşşahin, 2017; Yüce et al., 2016)
TNs	<i>Cyprinus carpio</i>	40	30.85	100-140	39.1-54.7	Sel	(Anonymous, 2020a; Cilbiz, Küçükçakara, et al., 2015; Yüce et al., 2016)
TNs	<i>Cyprinus carpio</i>	40	30.85	40-140	13.0-45.4	Sel	(Anonymous, 2020a; Aydın et al., 2016; Yüce et al., 2016)
TNs	<i>Cyprinus carpio</i>	40	30.85	130-160	50.8-62.6	Sel	(Anonymous, 2020a; Dereli et al., 2022; Yüce et al., 2016)
GNs	<i>Carassius gibelio</i>	25	9.7	32-90	8.8-24.7	Sel	(Balık et al., 2004; Cilbiz et al., 2014)
GNs	<i>Carassius gibelio</i>	25	9.7	32-90	8.7-24.6	Sel	(Balık et al., 2004; Cilbiz et al., 2014)
GNs	<i>Carassius gibelio</i>	25	9.7	40-100	12.3-30.7	Sel	(Aydın et al., 2018; Balık et al., 2004)
TNs	<i>Carassius gibelio</i>	25	9.7	100-140	24.9-34.9	Sel	(Balık et al., 2004; Cilbiz et al., 2014)
TNs	<i>Carassius gibelio</i>	25	9.7	100-140	27.2-38.1	Sel	(Balık et al., 2004; Cilbiz et al., 2014)
TNs	<i>Carassius gibelio</i>	25	9.7	100-120	23.8-28.5	Sel	(Balık et al., 2004; Korkmaz and Kuşat, 2014)
TNs	<i>Carassius gibelio</i>	25	9.7	40-100	12.0-30.1	Sel	(Aydın et al., 2018; Balık et al., 2004)
GNs	<i>Capoeta umbla</i>	20	23.3	56-76	26.0-35.3	Sel	(Anonymous, 2020a; Çoban et al., 2013; Gündüz et al., 2019)
TNs	<i>Capoeta baliki</i>	20	-	64-96	26.52-39.78	Sel	(Aydın et al., 2015)
GNs	<i>Esox lucius</i>	40	19.7	36-60	20.4-34.3	Sel	(Anonymous, 2020a; Balık, 2008; Balık et al., 2004)
GNs	<i>Esox lucius</i>	40	19.7	40-90	21.0-47.2	Sel	(Anonymous, 2020a; Balık et al., 2004; Cilbiz et al., 2017)
GNs	<i>Luciobarbus esocinus</i>	50	50	50-70	36.6-51.3	Holt	(Anonymous, 2020a; Eskandary et al., 2001; Yuksel et al., 2014)

**Table 1.** Continue.

Gear	Species	MCRS (cm)	LFM (cm)	MS (mm)	ML (cm)	Sel EM	Reference
GNs	<i>Oncorhynchus mykiss</i>	-	28.7	32-80	16.06-40.16	Sel	(Cilbiz, Yalim, et al., 2015; Wali et al., 2022)
GNs	<i>Rutilus rutilus</i>	-	16.8	32-80	12.5-30.4	Sel	(Hanol et al., 2015; Tarkan, 2002)
GNs	<i>Scardinius erythrophthalmus</i>	-	19.2	40-52	12.6-16.5	Holt	(Balik and Çubuk, 2001; Tarkan, 2002)
GNs	<i>Sander lucioperca</i>	26	25.6	40-52	21.2-27.5	Sel	(Anonymous, 2020a; Ozyurt et al., 2011)
GNs	<i>Sander lucioperca</i>	26	25.6	32-90	16.3-45.8	Sel	(Anonymous, 2020a; Cilbiz et al., 2022)
GNs	<i>Sander lucioperca</i>	26	25.6	34-70	15.9-32.9	Holt	(Anonymous, 2020a; Balik, 1999b; Ozyurt et al., 2011)
GNs	<i>Sander lucioperca</i>	26	25.6	40-52	20.6-26.8	Holt	(Anonymous, 2020a; Kiyaga, 2008; Ozyurt et al., 2011)
GNs	<i>Vimba vimba</i>	-	12.23	32-90	12.9-36.4	Sel	(Cilbiz, Apaydin Yağcı, et al., 2015; Erol, 2019)

Gear: GNs (Gill nets) and TNs (Trammel nets); MCRS: Minimum Conservation Reference Size as total length; LFM: Length at First Maturity; MS: Mesh Size (intended as stretched mesh length, range min-max); ML: Modal Length (range min-max); Sel EM (Estimation Method): SEL (SELECT), Holt (Holt, 1963); Reference for MCRS, LFM, gear selectivity

**Table 2.** Some biological reference points and linear regression parameters modelling the relationship between mesh length and modal length for the species targeted by gears.

Species	Net type	MCRS (cm)	LFM (cm)	MCRS / LFM ratio	Ideal mesh size (mm) Mean (CI 95%)	Linear regression parameters			
						Intercept (SE)	Mesh (SE)	p	R <sup>2</sup>
<i>C. carpio</i> *	GNs	40	30.84	1.29	114.4 (111.0 – 117.8)	35.62 (3.12)	1.95 (0.07)	<0.001	0.94
<i>C. carpio</i> *	TNs	40	30.84	1.29	112.2 (105.7 – 118.7)	20.58 (7.00)	2.25 (0.16)	<0.001	0.93
<i>C. gibelio</i> *	GNs	25	10.67	2.34	86.7 (83.8 – 89.7)	3.52 (2.47)	3.34 (0.13)	<0.001	0.97
<i>C. gibelio</i> *	TNs	25	10.67	2.34	93.5 (88.7 – 99.8)	-4.34 (9.35)	3.96 (0.32)	<0.001	0.90
<i>E. lucius</i> *	GNs	40	24.54	1.62	75.0 (72.8 – 77.1)	-4.26 (2.22)	1.99 (0.07)	<0.001	0.98
<i>S. lucioperca</i> *	GNs	26	22.00	1.18	52.9 (52.0 – 53.8)	1.42 (1.37)	1.95 (0.05)	<0.001	0.98
<i>A. grypus</i>	GNs	45	45	1.00	46.00	1.00 (5.00)	1.00 (9.00)	<0.001	1.00
<i>A. tarichi</i>	GNs	18	13.68	1.32	34.04	-0.002 (0.005)	1.89 (0.00)	<0.001	1.00
<i>C. antalyensis</i>	GNs	20	-	-	43.12	-0.001 (0.007)	2.16 (0.00)	<0.001	1.00
<i>C. umbla</i>	GNs	20	23.31	0.86	43.05	-0.006 (0.03)	2.15 (0.00)	<0.001	1.00
<i>L. esocinus</i>	GNs	50	50	1.00	68.28	0.018 (0.02)	1.37 (0.00)	<0.001	1.00
<i>O. mykiss</i>	GNs	-	28.7	-	57.17	0.006 (0.003)	1.99 (0.00)	<0.001	1.00
<i>R. rutilus</i>	GNS	-	16.8	-	42.48	-1.20 (9.10)	2.60 (4.10)	<0.001	1.00
<i>S. erythrophthalmus</i>	GNs	-	19.2	-	79.46	0.92 (1.28)	3.09 (0.9)	<0.001	1.00
<i>V. vimba</i>	GNs	-	12.23	-	26.66	-3.90 (2.01)	2.53 (7.93)	<0.001	1.00

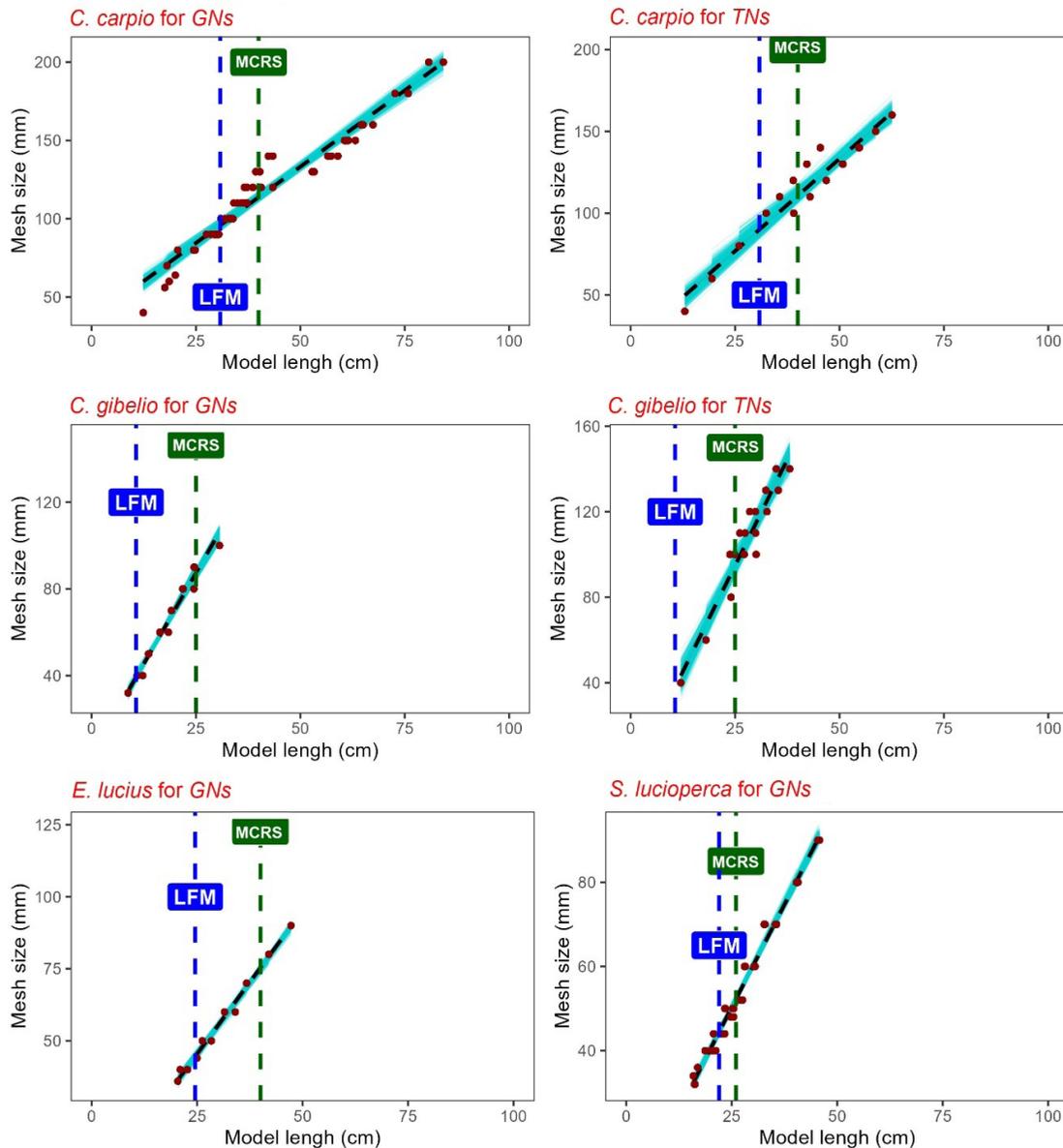
\* Ideal mesh size computed by using bootstrapped model for MCRS point; GNs: gillnets; TNs: trammel nets; LFM: length at first maturity; MCRS: Minimum conservation reference size.

*cioperca* is quite below the ideal mesh size for *C. carpio* with which it probably shares the same fishing grounds.

The selectivity indicator diagrams (Figure 3) used in this study which was developed by Lucchetti et al. (2020) for provide an immediate visual representation of whether a net can catch immature individuals. Figure 3 represents four catching scenarios. (i) The best scenario -catches mature individuals above the MCRS- (Figure 3- green area); (ii) discard scenario -catches mature individuals under the MCRS- (Figure 3- yellow area); (iii) worst case

scenario -catches immature individuals under the MCRS- (Figure 3- red area); bad case scenario catches immature individuals above the MCRS- (Figure 3- pink area).

The selectivity indicator diagrams were applied for only *C. carpio* (GNs-TNs), *C. gibelio* (GNs-TNs), *E. lucius* (GNs) and *S. lucioperca* (GNs) for which we have enough studies (Figure 4). A shift towards the upper right quadrant was observed in density graphs for all net types, so it indicates that the nets catch mature individuals above the MCRS (Figure 3, Figure 4).



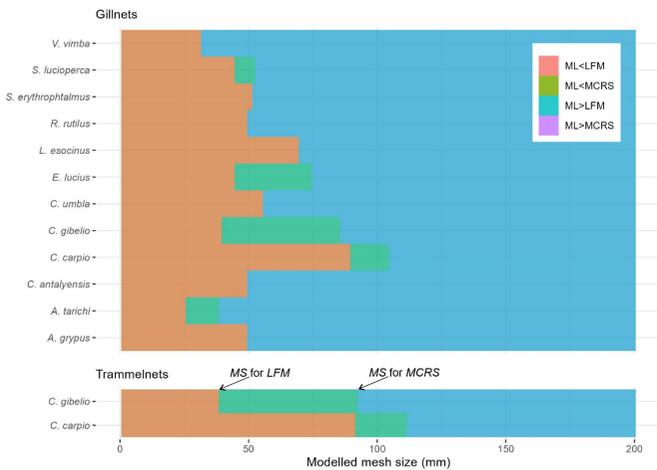
**Figure 1.** Relationship between mesh size and modal length in gillnets and trammel nets for the species that can be studied. (Shaded area: 95% confidence interval; solid cyan; black dotted line: linear regression; blue dotted line: LFM; green dotted line: MCRS obtained from the review).

## DISCUSSION

In the present study, in which selectivity features of GNs and TNs used in fishing 14 fish species in the inland fishery in Türkiye were evaluated, 34 set net selectivity studies were evaluated. The majority of the studies (75.7%) were carried out with GNs. Likewise, in a similar study conducted by Lucchetti et al. (2020), selectivity studies were found to be more common on GNs (65.0%) compared to TNs (35.0%). Specific to Türkiye, this is supposed to be related to cost. The cost to the fishermen of TNs made up by combining three different panels instead of GNs consisting of a single panel is higher; they prefer this cost only for fishing relatively bigger fish (e.g. *C. carpio*) which they have difficulty in fishing with GNs. The big *C. carpio* cause breakage of the net rope

because of fluttering while they are caught in the form of gilling on the side of GNs and in this way they can break away from the net, however, this is not the case when they are caught with TNs. In support of this argument, Balık (1996) reports that TNs are more efficient than GNs in fishing *C. carpio*.

As for the number of studies per species, 10 studies were carried out for *C. carpio*, seven for *C. gibelio*, four for *S. lucioperca*, two for *E. lucius*, two for *A. tarichi* and one study for each of the other species. This distribution is supposed to be due to the generality across the country of the species in general and their share in total production. According to Turkstat (2022) data, the first four species that are mostly fished in the inland fishery in Türkiye in 2001 and their production amounts are *A. tarichi* (9925), *C.*

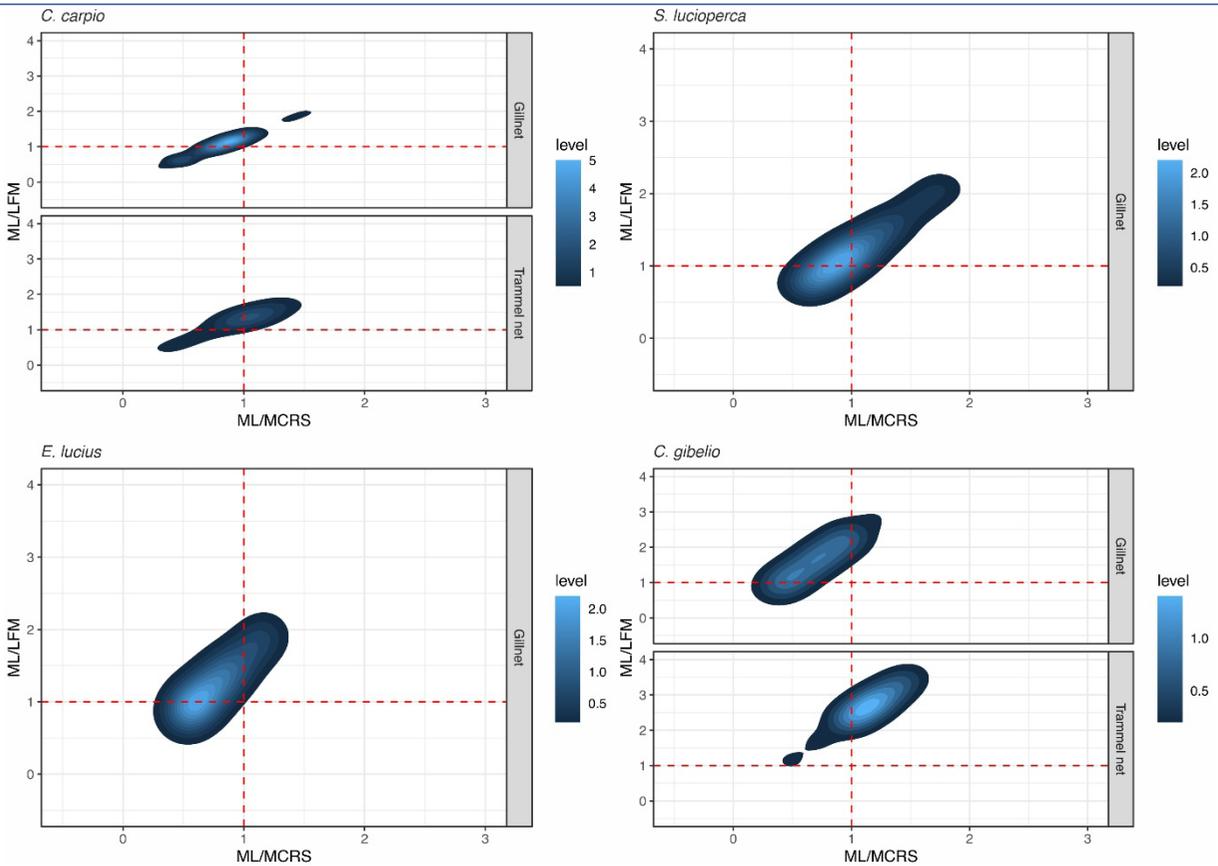


**Figure 2.** Ideal MS versus reference point comparison for different species (gillnets: upper; trammel nets: lower; MS: mesh size; LFM: length at first maturity; MCRS: Minimum conservation reference size).

*gibelio* (8039 tonnes), *A. boyeri* (6404 tonnes) ve *C. carpio* (3212 tonnes). In fishing *A. boyeri* in Türkiye, the coastal beach seine is used rather than GNs and TNs (Cilbiz et al., 2020).



**Figure 3.** Definition of theoretical selectivity indicator diagrams (Lucchetti et al., 2020) (ML: Modal length; LFM: Length at first maturity; MCRS: Minimum conservation reference size).



**Figure 4.** Selectivity indicator diagrams for the different species (ML: modal length; LFM: length at first maturity; MCRS: minimum conservation reference size).

Researchers mostly preferred *SELECT* method (69.7%) and partly Holt (1963) method (30.3%) in their studies for investigating *GNs* and *TNs* size selectivity. In general, it can be said that *SELECT* method was preferred in the more recent studies. This results from the fact that researchers prefer methods with relatively higher reliability within the framework of developing data evaluation technologies. According to Balık (2008), despite being one of the most widely used methods in set net selectivity, Holt (1963) is limiting; instead of it, the use of the *SELECT* method which is a statistical model estimating selectivity curve comparatively among different mesh size fishing and which provides an approach compatible with selectivity analysis has become widespread recently.

In this study, it was found that *LFM* for *A. grypus*, *B. bjoerkna* and *C. antalyensis* species is still not known and there is need for scientific studies for determining this value which is very important for sustainability of the species. On the other hand, there are species for which *MCRS* is not applied in commercial fishing although their *LFMs* are known (*C. gibelio*, *O. mykiss*, *R.utilus*, *S. erythropthalmus*, *V. vimba*, *A. boyeri*). There is no length limit in both amateur and commercial fishing for *C. gibelio*, *O. mykiss*, *A. boyeri* which are listed among the ecologically harmful (and potentially harmful species) for inland waters in Anonymous (2020b). In particular, although *C. gibelio* and *A. boyeri* that are among the first four species fished mostly in inland waters make significant contributions to the economy of both fishermen and the country, the fact that *MCRS* has not been declared for commercial fishing them may indicate that the ministry is not planning for the sustainability of natural stocks of these species.

The *MCRS* values declared for all species except *C. umbla* was found to be higher (*C. carpio*, *C. gibelio*, *E. lucius*, *S. lucioperca*, *A. tarichi*) than or equal (*A. grypus*, *L. esocinus*) to *LFM* value. In this context, the requirement that "the stock should find the chance of reproduction at least once before being caught for ensuring maintainability of the biomass" suggested by Beverton and Holt (1957) for a significant part of species is met in theory. However, whether determined legal regulations have been put into practice, in other words, whether fishermen comply with *MCRS* limits should be strictly inspected. Otherwise, no contribution should be expected from the criteria determined to the sustainability of natural fish stocks.

There is a selectivity study carried out by Balık and Çubuk, in 2001 for *B. bjoerkna*, however, it is a species with a very low economic value in the inland fishery in Türkiye and there is no legal length limit (*MCRS*) in fishing this species as it is often caught as a bycatch of other more important species; in addition, it was not subjected to further evaluation since no studies were found on the reproduction biology of the species.

When the relationship graphs given in Figure 1 are reviewed, it is seen that in general, the confidence interval for *TNs* is wider compared to that of *GNs* considering the studies carried out on the same species. This may be resulting from the higher spread value (*SR*) of *TNs* compared to that of *GNs* (from the fact that they capture a wider length range). In a study by Aydın et al. (2016), selectivity features of *GNs* and *TNs* with the same mesh size in fishing *C. carpio* were investigated and for nets with the

mesh sizes of 40, 60, 80 and 100 mm, *SR* values for *GNs* were reported to be 1.10, 1.65, 2.20, 2.75 cm in the same order while *SR* values for *TNs* were reported to be higher being 1.37, 2.06, 2.74 ve 3.43 cm respectively. Similarly, Lucchetti et al. (2020) also state that out of the set nets, especially *GNs* have high selectivity.

Under the legislation regulating commercial fisheries in Türkiye (Anonymous, 2020a), minimum mesh size application in inland waters is in place for removing certain lengths of certain species from the catch composition. Minimum mesh size that can be used by fishers in fishing is determined by local administrative units. At this point, because the legal practice does not follow a strategy that is based on a scientific foundation, different mesh sizes are declared in different fishing places for the same species and this leads to discomfort among the fishers. This study has a lot of promise for overcoming this handicap. When looked at other countries where composition of species is wide, it is observed that a standard ideal mesh size cannot be determined as is the case in Türkiye. For example, in Greece, the mesh size of nets used by professional fishermen in inland fishing is required to be longer than 20 mm (bar length), however, this rule does not apply to *C. carpio* (55 mm), *Alburnus sp.* and *Atherina boyeri* (15 mm) (Petriki et al., 2014).

Consequently, it was found that *MCRS* values determined by the Ministry in the inland fishery in Türkiye are substantially greater than *LFMs* of species. In this context, it can be asserted that the criterion that species should find the opportunity of reproducing at least once before being fished is met, and that sustainability of fishery is significantly guaranteed. On the other hand, fishing gears used for fishing were found to be typically tending to fish mature individuals on *MCRS*. The major problem in the use of set net in the inland fishery in Türkiye is that different ideal mesh sizes are determined for different fish species that are in the same fishing place. A mesh size that is ideal for a species may have the potential of fishing individuals below *LFM* for another species in the same fishing grounds. For resolution of this problem, fishery management authorities are advised to determine vulnerable species (that are exposed to overfishing, economically much more valuable, among endangered species, of high importance in ecological terms) within the frame of fishing place-based on-site management and to determine mesh width based on the objective of protecting them to a minimum extent. This problem which is not very likely to be resolved with conventional fishery management focusing on single or targeted species is likely to be resolved with the Ecosystem Approach to Fisheries Management (*EAFm*) specifically recommended for inland fisheries by FAO (2021).

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## Where Have You Been Hiding? Re-Emergence of *Squatina oculata* in the Sea of Marmara, with a Review of Captures in Turkish Seas

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### ABSTRACT

On February 27, 2018, a female smoothback angelshark, *Squatina oculata*, was incidentally captured in a gill-net fishery off the Mudanya coast (southeastern Sea of Marmara). An opportunistic photographic record confirmed the emergence of *S. oculata* in the Sea of Marmara, where its status in the area is considered questionable because of its absence for many years in the region. The current opportunistic records are valuable data sources, which can provide complementary information to fill the knowledge gaps in the life histories of rare, rarely caught, or endangered sharks and rays.

**Keywords:** *Squatina oculata*, Turkish Sea, emergence, endangered, opportunistic

### INTRODUCTION

Angelsharks (Squatiniformes: Squatinidae) are represented by 22 species and *Squatina oculata* Bonaparte, 1840, commonly known as the smoothback angelshark, emerged in the Eastern Atlantic, from Senegal to Ghana coast, and in the Mediterranean Sea (Ebert et al., 2021). Although its present emergence in the Mediterranean Sea has long been well-recognized (Miller, 2016; Serena et al., 2020; Barone et al., 2022), *S. oculata* is considered a rare and one of the least-known species of Mediterranean sharks for which data collection is clearly required owing to knowledge gaps (Zava et al., 2016; Barone et al., 2022; Zava et al., 2022). First accounts of the emergence of smoothback angelshark in Turkish waters were mentioned in the inventories of Slastenenko (1955–1956; cited in Bilecenoğlu et al., 2002, 2014), Akşiray (1987), and Bauchot (1987). Despite being reported in the Sea of Marmara for the first time in the 1950s and then reported from the same region in the early 1990s (Slastenenko, 1955–1956; Meriç, 1994; both were cited in Bilecenoğlu

et al., 2002, 2014), the species has not been reported in several bycatch inventories (Bayhan et al., 2006; Bök et al., 2011) or general ichthyological inventories (Karakulak et al., 2017; Daban et al., 2021; ÇŞİDB-TUBİTAK-MAM, 2021) of the Sea of Marmara, which were previously published. Although the emergence of *S. oculata* in the region was also reported by Eryılmaz and Meriç (2005), this review study is based on the ichthyological report by Meriç (1994; cited in Bilecenoğlu et al., 2002). Due to the absence of a recent record of smoothback angelsharks from the region, the emergence status of *S. oculata* was considered questionable (Kabasakal, 2022); thus, it requires confirmation. In the present study, the re-emergence of the smoothback angelshark in the Sea of Marmara after nearly 30 years is reported, and a review of captures of the species in Turkish waters is provided.

### MATERIAL AND METHODS

The present record of *S. oculata* was obtained following data mining to extract shark-specific

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information (printed or digital articles, social media posts or films, etc.) as part of an ongoing data acquisition survey to regularly update the information on sharks of Turkish seas, which has been continuing since 2000 through monthly screening of published media (2000–2009 records) and Internet media (post2009 records). While scanning and extracting data shared by digital content owners on the Internet or social media, ethical rules proposed by Monkman et al. (2017) were followed using the data in question. Furthermore, care was taken to not violate the rights of content owners and show the faces of people in the visual content to prevent revealing their identities. Information regarding the present smooth angelshark is available on the website of a mainstream newspaper of Turkish media (Türk, 2018).

Species identification follows the descriptions by Miller (2016), Ebert et al. (2021), and Barone et al. (2022). Taxonomic nomenclature follows that reported by Froese and Pauly (2023). Regarding the sampling methodology, the present study is a usual practice of opportunistic sampling, of which opportunistic records are not the direct results of scientific fisheries surveys but are acquired from printed or digital media, social media, logs of naturalists, or recreational fishermen (Grant et al., 2022; Hiddink et al., 2023). Previous captures of *S. oculata* in Turkish waters were extracted from relevant references presented in Table 1.

## RESULTS AND DISCUSSION

On February 27, 2018, a female smoothback angelshark (Figure 1) was incidentally captured in a gill-net fishery off the Mudanya coast (southeastern Sea of Marmara; Figure 2). The present specimen was identified as *S. oculata* based on the following characteristics: a ray-like shark species with a strongly dorsoventrally flattened body, a very wide head, widened pectoral and dorsal fins, and no anal fin; however, the head is separated from the pectoral fins with very deep insertions. The origin of the first dorsal fin is located well behind the free rear tips of the pelvic fins, a primary descriptive characteristic of the three species of angelsharks occurring in the Mediterranean Sea (Figure 1). The area between the eyes is strongly concave. The lower lobe of the caudal fin is larger than the upper lobe. Nasal barbels are quite simple; however, because of the resolution and sight angle of the present image, no details of the nasal barbels, defined as “moderately fringed” in the literature, can be observed in the photo of the present specimen. Large spiracles are observed behind the eyes. The mouth is terminal and wide, and the teeth on the upper and lower jaws are small, pointed, and with one cusp. The above description of the present specimen is in agreement with the published descriptions of *S. oculata* (Miller, 2016; Ebert et al., 2021; Barone et al., 2022).

Captures of *S. oculata* reported from the Sea of Marmara and off the Turkish coasts of the Aegean Sea and the Mediterranean are presented in Table 1. In this table, 22 smoothback angelsharks have been incidentally captured in Turkish waters between pre1956 and 2023. The majority of smoothback angelsharks have been captured in Turkish Mediterranean waters (n = 13; 59.09%), followed by Turkish Aegean waters (n = 6; 27.2%) and Sea of Marmara (n = 3; 13.6%). The size (TL) of the captured smoothback angelsharks varied between approximately 24 and 120 cm, and the

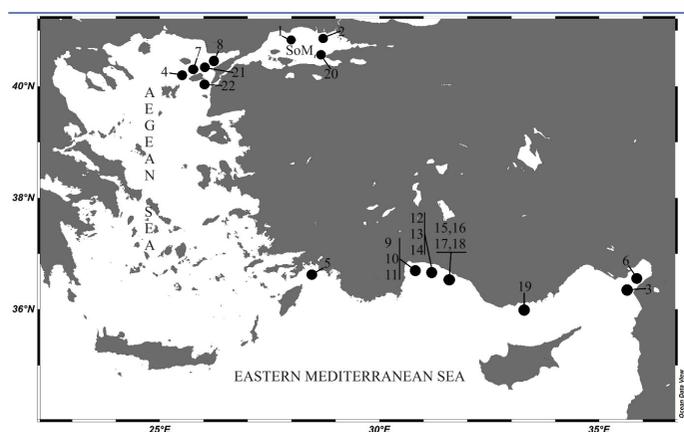
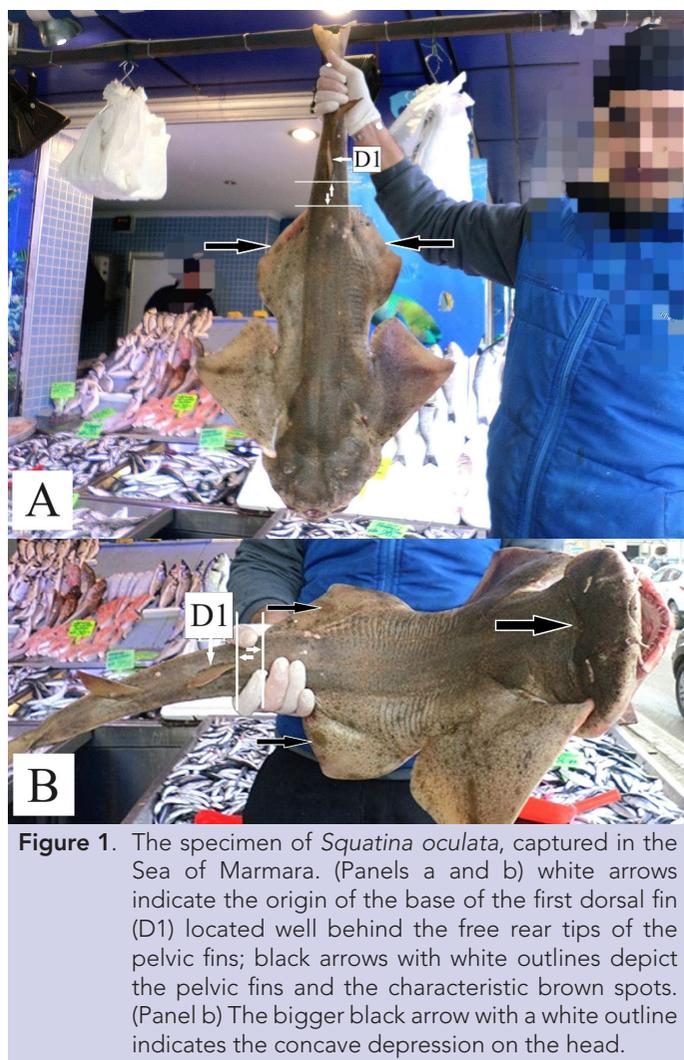
**Table 1.** Review of captured specimens of *Squatina oculata* in Turkish waters.

No	Date	Locality	TL (cm)	W (g)	Sex	Depth (m)	Fishing gear	Remarks	Reference
1	Pre1956	SoM	-	-	-	-	-	-	Slattenenko (1955–1956; cited in Bilecenoğlu et al., 2002, 2014)
2	Pre1994	SoM	-	-	-	-	-	-	Meriç (1994; cited in Bilecenoğlu et al., 2002)
3	Pre1996	Iskenderun Bay, northeastern MS	75.6	4,000	-	60	BT	Captured over mixed muddy-sandy bottom off Karataş	Başusta ve Erdem (2000)
4	Pre2002	Gökçeada, northern AE	-	-	-	-	-	-	Kabasakal (2002)
5	Pre2002	Fethiye, southern AE	-	-	-	-	-	-	Kabasakal (2002)
6	Pre2002	Iskenderun Bay, northeastern MS	-	-	-	-	-	-	Kabasakal (2002)
7	Jul 1997	Gökçeada, northern AE	30	180	♂	-	BT	Captured over sandy bottom mixed with small pebbles and vegetation of <i>Posidonia oceanica</i>	Kabasakal & Kabasakal (2004)

Table 1. Continue.

No	Date	Locality	TL (cm)	W (g)	Sex	Depth (m)	Fishing gear	Remarks	Reference
8	Sep 1999	Gökçeada, northern Aegean Sea	95	6,000	♀	-	BT	Captured over sandy bottom	Kabasakal & Kabasakal (2004)
9	Aug 2009	Bay of Antalya, eastern MS	80	4,500	-	100	BT	-	Özgür Özbek & Kabasakal (2022)
10	Spring 2010	Bay of Antalya, eastern MS	50	850	-	100	BT	-	Özgür Özbek & Kabasakal (2022)
11	Spring 2010	Bay of Antalya, eastern MS	88	5,550	♀	50	BT	A total of seven developing embryos, with a total weight of 500 g, were found in uteri. Photographic documentation of the intra-uterine embryos is available in the relevant reference	Özgür Özbek & Kabasakal (2022)
12	Spring 2010	Bay of Antalya, eastern MS	59	1,600	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
13	Spring 2010	Bay of Antalya, eastern MS	52	1,000	-	100	BT	-	Özgür Özbek & Kabasakal (2022)
14	Winter 2010	Bay of Antalya, eastern MS	67	1,700	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
15	Winter 2010	Bay of Antalya, eastern MS	69	1,800	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
16	Winter 2010	Bay of Antalya, eastern MS	66	2,000	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
17	Winter 2010	Bay of Antalya, eastern MS	88	5,500	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
18	Winter 2010	Bay of Antalya, eastern MS	24	71	-	50	BT	-	Özgür Özbek & Kabasakal (2022)
19	4 Nov 2017	Aydıncık coast, eastern MS	72.6	3,450	♀	65	BT	Captured over sandy bottom	Ergüden et al. (2019)
20	27 Feb 2018	Mudanya coast, southwestern SoM	-	-	-	-	GN	Displayed in the fishmonger	Present study
21	22 Mar 2018	Gökçeada, northern Aegean Sea	87.5	5,536	♀	110	BT	Symmetrically distributed developing oocytes (n = 6) ranging from 55.22 to 59.55 mm, were found in ovaries	Yığın et al. (2019)
22	8 Aug 2023	Gökçeada, northern Aegean Sea	ca. 120	-	♀	224–300	TN	Released alive	Kabasakal (2023, in press)

SoM, Sea of Marmara; AE, Aegean Sea; MS, Mediterranean Sea; BT, bottom trawl; GN, gill net; TN, trammel net



weight varied between 71 and 6,000 g (Table 1). The collated data by Zava et al. (2022), between September 2005 and March 2021, revealed that 34 smoothback angelsharks have been either captured or sighted from several regions of the Mediterranean Sea. When the study results and data by Zava et al. (2022) are combined, the number of confirmed records of *S. oculata* in the Mediterranean Sea rises to 56, 39.28% of which have been captured in Turkish waters.

During the second phase of the extensive MEDITS survey performed in northern Mediterranean waters between 2012 and 2015, no *S. oculata* was captured (Follesa et al., 2019). Furthermore, during the first phase of the MEDLEM project, which is aimed at collating data on the large elasmobranch species of the Mediterranean Sea between 1666 and 2017, *S. oculata* is considered a "rarely observed" shark species (Mancusi et al., 2020). During the second phase of the MEDLEM, covering 2017–2022, no smoothback angelshark has been recorded in the area (Gallo et al., 2022). Therefore, the smoothback angelshark sighted in the trammel net fishery on August 8, 2023, off the island of Gökçeada (sp No. 22 in Table 1; Kabasakal, 2023), is a recent record of species in the Mediterranean Sea, without ruling out the rarity of *S. oculata*. Pregnant females captured in the Bay of Antalya and off the island of Gökçeada (sp Nos. 11 and 21, respectively, in Table 1) indicate that *S. oculata* reproduction may simultaneously emerge in remote regions of the northern Aegean Sea and Turkish Mediterranean waters. The emergence of pregnant females provides further justification for the International Union for Conservation of Nature Shark Specialists Group's declaration of the Tracean Sea Shelf (northern Aegean Sea) and Bay of Antalya (eastern Mediterranean) important shark and ray areas (Jabado et al., 2023).

Recent records of *S. oculata* in Turkish seas are mostly composed of individuals captured after 2000 ( $n = 19$ ; 86.36%; Table 1). Similarly, Zava et al. (2022) reported that all 34 smoothback angelsharks were either captured or sighted in 2005 and thereafter. Again, when the results of this study and those of Zava et al. (2022) are combined, the majority of contemporary records of smoothback angelsharks ( $n = 53$ ; 94.64%) have either been captured or sighted in the last 23 years, which corresponds to 2.3 individuals per year. Although the number of smoothback angelsharks per annum reflects the rarity of the species, the paucity of records may be due to the absence of reporting captures or sightings. Therefore, the available number of records may increase by unraveling the retrospective unpublished smoothback angelshark captures. For example, despite the present capture of a smoothback angelshark on February 27, 2018, the discovery of this unpublished record occurred following an Internet search performed on December 14, 2023, which emphasizes the importance of retrospective data searches with novel investigation methods and digital tools.

In recent years, a notable increase in the use of digital tools, such as digital media, and social media, among others, in searching for rare elasmobranchs and in the number of studies focusing on captures and/or sightings of these species has been reported (Bengil, 2020; Kabasakal & Bilecenoğlu, 2020; Grant et al., 2022; Hiddink et al., 2023; Kabasakal, 2023). A quick review of the contents of such research articles reveals a wide spectrum of topics, such as providing complementary data on the distribution of

well-recognized elasmobranchs (Bengil, 2020) or rare and large sharks (Kabasakal and Bilecenoğlu, 2020) in Turkish waters, filling the knowledge gaps of the distribution range of an endangered elasmobranch species (Grant et al., 2022), or determining spatial and temporal variations of the distribution and abundance of a certain elasmobranch (Hiddink et al., 2023). Compared with traditional systematic scientific sampling, opportunistic methodologies obviously suffer from several drawbacks, such as uncertainties (e.g., fisher's statement-dependent information) and weaknesses (e.g., fisher's data cannot be representative of the true periodicity of fishing days). However, as emphasized by Tsikliras and Dimarchopoulou (2021), the current opportunistic records are considered valuable data sources; these can provide complementary information to fill the knowledge gaps in the life histories of rare, rarely caught, or endangered sharks and rays. Finally, the identifying a species from the photo provided, which is also the case for the present study, is considered a confirmed emergence of a fish species in a given region (Kovačić et al., 2020).

## CONCLUSIONS

As a consequence, the contemporary emergence of *S. oculata* in the Sea of Marmara, where the status of the species in the region is considered questionable due to its absence for many years in the area (Kabasakal, 2022), is confirmed based on an opportunistic photographic record (Figure 1). Being a "critically endangered" elasmobranch species of the Mediterranean Sea (Ferretti et al., 2016) and being included in the Annex II of the SPA/BD protocol covered by GFCM/36/2012/3 and GFCM/42/2018/2 recommendations (Barone et al., 2022), *S. oculata* is also a protected species in Turkish seas (Resmi Gazete, 2018). However, even the protected species can be incidentally captured in commercial fisheries, which is also the case for the present smoothback angelshark. Since the data in question can often be hidden due to fear of punishment, social media posts or digital news reports of such hidden captures may be used as an efficient and complementary data-providing tool for monitoring the bycatch of endangered and protected species in commercial fisheries.

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**Ethics Committee Approval:** Since no experimental animal was used in the submitted study, approval of the ethics committee is not required.

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After the Conclusion section and before references list, information regarding conflict of interest, financial disclosure, ethics committee approval and acknowledgement are given. These information are to be provided in the author form which must be submitted together with the manuscript.

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**Table 1.** Limitations for each manuscript type

Type of manuscript	Page	Abstract word limit	Reference limit
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Short Communication	≤5	250	20

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Figures, graphics, and photographs should be submitted as separate files (in TIFF or JPEG format) through the submission system. The files should not be embedded in a Word document or the main document. When there are figure subunits, the subunits should not be merged to form a single image. Each subunit should be submitted separately through the submission system. Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in

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When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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## References

While citing publications, preference should be given to the latest, most up-to-date publications. If an ahead-of-print publication is cited, the DOI number should be provided. Authors are responsible for the accuracy of references. List references in alphabetical order. Each listed reference should be cited in text, and each text citation should be listed in the References section. The reference styles for different types of publications are presented in the following examples.

### Reference Style and Format

Aquatic Sciences and Engineering complies with APA (American Psychological Association) style 6<sup>th</sup> Edition for referencing and quoting. For more information:

- American Psychological Association. (2010). Publication manual of the American Psychological Association (6<sup>th</sup> ed.). Washington, DC: APA.
- <http://www.apastyle.org>

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### Citations in the Text

Citations must be indicated with the author surname and publication year within the parenthesis.

If more than one citation is made within the same parenthesis, separate them with (;).

## Samples:

### **More than one citation;**

(Esin et al., 2002; Karasar, 1995)

### **Citation with one author;**

(Akyolcu, 2007)

### **Citation with two authors;**

(Sayiner & Demirci, 2007)

### **Citation with three, four, five authors;**

First citation in the text: (Ailen, Ciembrune, & Welch, 2000)

Subsequent citations in the text: (Ailen et al., 2000)

### **Citations with more than six authors;**

(Çavdar et al., 2003)

## Major Citations for a Reference List

Note: All second and third lines in the APA Bibliography should be indented.

- **A book in print:** Baxter, C. (1997). *Race equality in health care and education*. Philadelphia: Ballière Tindall. ISBN 4546465465
- **A book chapter, print version:** Haybron, D. M. (2008). Philosophy and the science of subjective well-being. In M. Eid & R. J. Larsen (Eds.), *The science of subjective well-being* (pp. 17-43). New York, NY: Guilford Press. ISBN 4546469999
- **An eBook:** Millbower, L. (2003). *Show biz training: Fun and effective business training techniques from the worlds of stage, screen, and song*. Retrieved from <http://www.amacombooks.org/> (accessed 10.10.15)
- **An article in a print journal:** Carter, S. & Dunbar-Odom, D. (2009). The converging literacies center: An integrated model for writing programs. *Kairos: A Journal of Rhetoric, Technology, and Pedagogy*, 14(1), 38-48.
- **An article with DOI:** Gaudio, J. L. & Snowdon, C. T. (2008). Spatial cues more salient than color cues in cotton-top tamarins (*saguinus oedipus*) reversal learning. *Journal of Comparative Psychology*, <https://doi.org/10.1037/0735-7036.122.4.441>
- **Websites - professional or personal sites:** The World Famous Hot Dog Site. (1999, July 7). Retrieved January 5, 2008, from <http://www.xroads.com/~tcs/hotdog/hotdog.html> (accessed 10.10.15)

- **Websites - online government publications:** U.S. Department of Justice. (2006, September 10). Trends in violent victimization by age, 1973-2005. Retrieved from <http://www.ojp.usdoj.gov/bjs/glance/vage.htm> (accessed 10.10.15)
- **Photograph (from book, magazine or webpage):** Close, C. (2002). *Ronald*. [photograph]. Museum of Modern Art, New York, NY. Retrieved from [http://www.moma.org/collection/object.php?object\\_id=108890](http://www.moma.org/collection/object.php?object_id=108890) (accessed 10.10.15)
- **Artwork - from library database:** Clark, L. (c.a. 1960's). *Man with Baby*. [photograph]. George Eastman House, Rochester, NY. Retrieved from ARTstor
- **Artwork - from website:** Close, C. (2002). *Ronald*. [photograph]. Museum of Modern Art, New York. Retrieved from [http://www.moma.org/collection/browse\\_results.php?object\\_id=108890](http://www.moma.org/collection/browse_results.php?object_id=108890) (accessed 10.10.15)

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When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be canceled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

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