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Research Article

A biological and ecological study of the invasive pufferfish *Torquigener hypselogeneion* (Bleeker 1852) [conspecific *Torquigener flavimaculosus* Hardy & Randall, 1983] in the Eastern Mediterranean

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Abstract

The highly toxic orange-spotted toadfish *Torquigener hypselogeneion* (Bleeker 1852) [conspecific *Torquigener flavimaculosus* Hardy & Randall, 1983] is now a very common invasive fish in the Eastern Mediterranean. Its small size, well under 20 cm, may have concealed the danger it represents, and little is known about its biology or ecology. Here, the spawning seasons, gonado- and hepato-somatic index and condition factors of *T. hypselogeneion* from 3 locations of the Eastern Mediterranean are presented, based on a total of 1360 individuals sampled, i.e., 216 from Finike, 817 from Fethiye (both Turkey), and 327 from Cyprus. Our results show that *T. hypselogeneion* is a carnivorous species that forages on sandy bottoms, with a preference for small invertebrates, especially the small invasive gastropod *Cerithium scabridum*, crustaceans (hermit crabs, other crabs and barnacles), and sea urchins; however, at least in some localities, they appear to forgo eating during their peak reproductive period. The parameters of the von Bertalanffy Growth Function for *T. hypselogeneion* in the Eastern Mediterranean were: asymptotic length = 17.4 cm (total length; TL) and K = 0.96 year^{-1}, implying a longevity of about 4 years, while the mean length at first maturity was about 10 cm (TL) for both sexes. An average-sized adult female (13 cm TL, 45.7 g live weight) was found to contain 1,250 eggs per gram body weight. Based on its high invasiveness and negative impacts to ecology of the Eastern Mediterranean and the human health, we suggest that *T. hypselogeneion* should be listed as a priority invasive species and that its population closely monitored within the Mediterranean Sea.

Key words: Invasive Alien Species (IAS), diet, growth, reproduction, spawning season, Tetraodontidae

Introduction

As we transition into a time of declining global marine ecosystem health, increasingly affected by human-induced changes (Steffen et al. 2011; Steffen 2021), the ecosystem services that permitted human population growth and geographic expansion of humans are now under threat, and these threats will have to be faced and mitigated. The threats facing marine ecosystems include the impacts of alien species, overexploitation, pollution and climate change (Arthington et al. 2016).

The Suez Canal is responsible for most alien species records in the Eastern Mediterranean (Galil et al. 2017), as it is man-made and connects the tropical Red Sea with the temperate Mediterranean Sea. Alien species are increasingly being reported in the Eastern Mediterranean Sea and have increased by 40% within the last decade alone (Zenetos et al. 2022). Proximity to the Suez Canal correlates to a higher number of alien species records (Ulman et al. 2019). Some alien species may bring some benefits to their new environments, for example by supporting new fisheries, for example, the fishery for the sea snail *Rapana venosa* in the Black Sea; Demirel et al. 2021, the blue crab *Callinectes sapidus* throughout the Mediterranean and Randall's threadfin bream *Nemipterus randalli*, the latter now supporting a longline fishery in the Eastern Mediterranean (Mavruk et al. 2020; Mutlu et al. 2023). Alien species are called 'invasive' when their introduction and/or spread by human action outside their natural distribution threatens biological diversity, food security, and human health and well-being (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services IPBES 2022 website: https://ipbes.net/glossary/invasive-alien-species). Specifically, this widely accepted definition states that alien species are deemed invasive when they expand into and modify ecosystems to which they have been introduced.

Alien marine species not targeted by fisheries are studied much less than their targeted counterparts, but each alien species needs to be assessed to understand their impacts on the biodiversity, ecosystem function, ecosystem services, as well as human health and socio-economic wellbeing, before managers can understand if a response is required and possible (Katsanevakis et al. 2014; Leuzinger and Rewald 2021; Salimi et al. 2021).

There are nearly 200 different species of pufferfishes around the world (Ulman et al. 2021; see also Froese and Pauly 2022). Of these, 29 belong to the genus *Torquigener* Whitley, 1930, all of which are native to the Indo-Pacific region (see FishBase, www.fishbase.org). The orange-spotted toadfish *Torquigener hypselogeneion* (Bleeker 1852) (Figure 1) is native to the Indo-Pacific region from Knysna, South Africa, Samoa in the east and Japan in the north (Froese and Pauly 2022). *T. hypselogeneion* is the only 'Lessepsian' species of this genus found in the Mediterranean. It was first reported in the Mediterranean in 1987 from Israeli waters (Golani et al. 1987), but was not found to have established self-sustaining populations until 2002 in Fethiye, southwestern Turkey (Bilecenoğlu 2003).

Since then, *T. hypselogeneion* has also spread to Greece, Syria, Egypt, Cyprus and Libya (Figure 2; Corsini-Foka et al. 2006; Zenetos et al. 2007; Sabour et al. 2015; Farrag et al. 2016; Al Mabruk et al. 2018). It is the second smallest pufferfish found in the Mediterranean, after *Tylerius spinosissimus*, with a mean size around 10–12 cm; a "single, exceptionally large specimen of 18.5 cm TL was collected in Haifa Bay, Israel" (Golani et al. 2021). A recent molecular and morphological examination of this species now suggests that *Torquigener flavimaculosus* is a junior synonym of *Torquigener hypselogeneion* (Bilecenoğlu and Yokeş 2022).

This orange-spotted toadfish *T. hypselogeneion* may be seen as 'cute', with bright emerald eyes lined with gold. It is a demersal species that generally hovers just a few

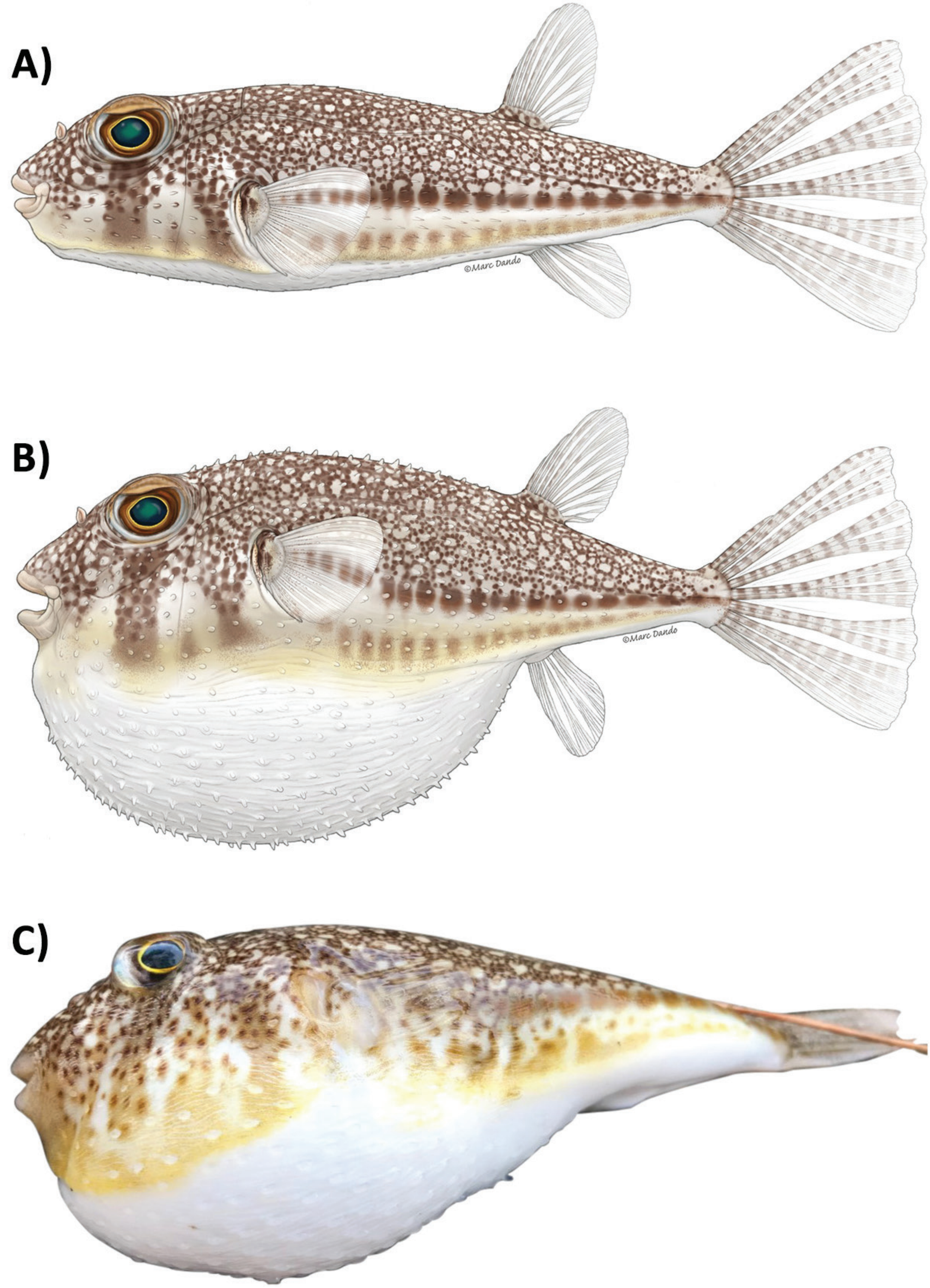

Figure 1. The orange-spotted toadfish *Torquigener hypselogeneion* (Bleeker 1852) [conspecific *Torquigener flavimaculosus* Hardy & Randall, 1983], Family Tetraodontidae. **A:** Lateral view, normal shape; **B:** The same fish, puffed (both illustrations by Marc Dando). **C:** Photo of a specimen from Fethiye, Turkey (June 2021; photo by A. Ulman).

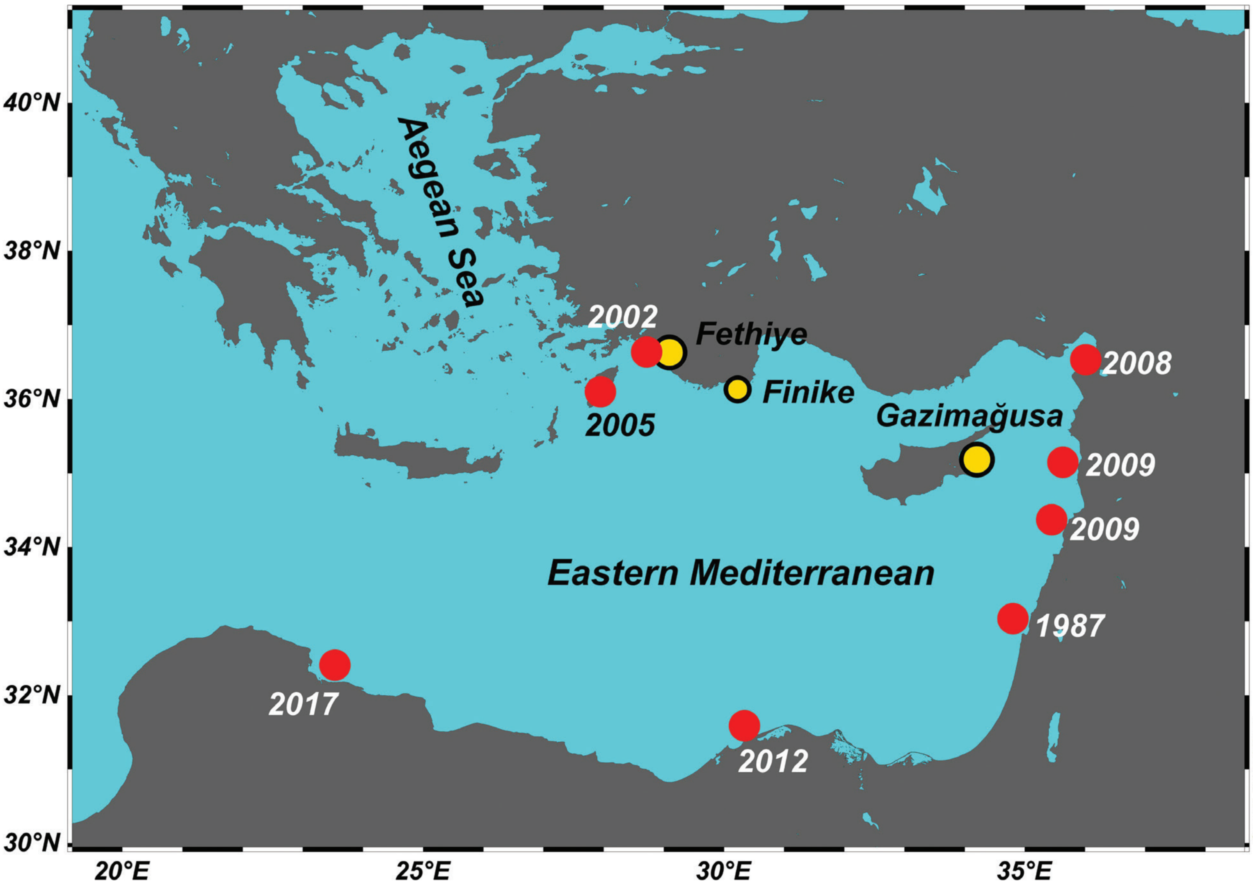

Figure 2. Distribution and years of the first records of orange-spotted toadfish *Torquigener hypselogeneion* (conspecific yellowspotted puffer *Torquigener flavimaculosus*) in the Mediterranean (1987–2017: red dots), and locations of study sites (2017–2021; yellow dots). The coordinates for the points are provided in Suppl. material 4.

centimeters above the seafloor at depths from 3 to 75 m (Mutlu et al. 2021). Its brown mottled dorsal side serves as a perfect camouflage viewed from above on a sandy or gravel bottom habitat, where it is most often found. It is a mesoconsumer that commonly forages invertebrates in the sand (Chartosia et al. 2021). Thus, it is a carnivore that consumes of mix of herbivores and small carnivores. A 2014–2015 stock assessment of its population in Finike Bay, Turkey, suggested it had a maximum biomass of 2 $kg{\cdot}km^2$, and an average abundance of 150 $individuals{\cdot}km^2$ (Mutlu et al. 2021).

T. hypselogeneion contains an extremely high content of tetradotoxin (TTX), a poison which can be fatal to humans. Pufferfish are considered unsafe for human consumption when they contain over 2 $mg{\cdot}kg^{-1}$ (Noguchi and Ebesu 2001; Katikou 2019), or > 2.2 µg/g of TTX (Bane et al. 2014), *T. hypselogeneion* contains TTX at concentrations of over 15, 5, 12, 7 and 35 µg/g in its muscle, gonad, intestine, liver and skin, respectively (Kosker et al. 2018), which makes it a particularly dangerous invasive species (Martinou et al. 2018; Chartosia et al. 2021). Its high TTX content probably protects *T. hypselogeneion* from many would-be predators, and so far, only the much larger and very common invasive silver-cheeked toadfish *Lagocephalus sceleratus* (Gmelin, 1789) has been demonstrated to prey on *T. hypselogeneion* over a dozen times and survive (Ulman et al. 2021).

According to the Aquatic Species Invasiveness Screening Kit (AS-ISK) of five pufferfish species from the Muğla province region in Turkey, *T. hypselogeneion* scored 31, with species needing to pass the threshold score of 18.5 to be classified as invasive (Filiz et al. 2017). Its higher score was attributed to their invasiveness elsewhere, their undesirability or persistence traits, high dispersal mechanisms and high climate match, tolerance of a wide range of environmental conditions, flexibility in utilizing food resources, high fecundity, small size at maturity, and high reproductive efforts. Their abundances are exploding in south-western Turkey, especially in sandy areas <15 m in depth, for example, one site regularly checked by the first author (Kidrak Beach, Olüdeniz, Fethiye) has over 100 individuals per 10 $m^{2,}$ and fishers in the region are also saying it is now the most abundant pufferfish. Based on the definition of invasive species, their spreading and high abundances in certain areas combined with a threat to human and animal health if ingested should warrant its classification as an invasive species.

Most Mediterranean prior research relates to either new records (Golani 1987; Bilecenoğlu 2003, 2005; Corsini-Foka et. al. 2006; Zenetos et al. 2007; Ergüden and Gürlek 2010; Sabour et al. 2015; Farrag et al. 2016; Al Mabruk et al. 2018), maturity and reproduction (Çek-Yalnız et al. 2017; Ramadan and El-Hafawy 2019), length-weight relationships (Edelist 2014; Ergüden et al. 2015; Ayas et al. 2019; Ergüden et al. 2020), diet composition and trophic level (Chartosia et al. 2021), stock assessment (Mutlu et al. 2021), and its TTX content (Kosker et al. 2018). This study presents the results of new research on the growth, diet and reproduction of *T. hypselogeneion* using a much wider spectrum of samples and more comprehensive analyses than most prior studies, in order to improve on the biological and ecological understanding of this dangerous invasive species.

Material and methods

The identification of species was performed based on identification keys provided by Hardy (1983), Hardy and Randall (1983), Hardy (1984) and Bilecenoğlu and Yokeş (2022). Status of the species is controversial since taxonomical investigation performed by Bilecenoğlu and Yokeş (2022) suggests that *T. flavimaculosus* and *T. hypselogenion* are conspecific, making *T. flavimaculosus* a junior synonym of *T. hypselogeneion*. As this is the most recent investigation into its identity, the new suggested name is adopted here.

Samples were collected from Finike (36.295 N; 30.141 E), Antalya Province, Turkey by Ersönmez (2019; see Suppl. material 1); from Fethiye (36.640 N, 29.127 E), Muğla Province, Turkey by Aylin Ulman (see Suppl. material 2), and from Gazimağusa (35.121N, 33.939 E), Cyprus by Hasan Deniz Akbora and Burak Ali Çiçek (see Suppl. material 3). Table 1 provides details on the sampling localities, number of samples, collection dates and analyses used according to locality in this study.

Table 1. Locations in Turkey (T) and Cyprus (C) and months/year of sampling specimens of *Torquigener hypselogeneion* for studies of their Gonadosomatic Index (GSI), Hepato-Somatic Index (HSI), Condition Factor (CF), Stomach Fullness (SF), and Fecundity (F).

Locality	n	Sampling dates	GSI	HSI	CF	SF	F
Fethiye[a)] (T)	815	3/2020-8/2021	X	X	X	X	X
Finike (T)	216	3/2017-2/2018	X	X	X	–	–
Gazimağusa (C)	326	5/2020-10/2021[b]	X	X	X	X	–

[a)] Note that Fethiye was also the site where the length-frequency data used from the estimation of growth parameters were collected, along with the individuals used for histological, fecundity and diet studies; [b)] August and September 2020 were not covered in Cyprus due to Covid restrictions; thus, August and September 2021 were sampled instead to complete an annual data set.

Samples of *T. hypselogeneion* were purchased from small-scale commercial fishers using gill and trammel nets, hooks and lines in Fethiye (Turkey) and Gazimağusa (Cyprus). The price given was 5 Turkish Lira (TRY) for each fish (≈ 0.37 USD). In Finike, fishers were paid a total of 500 TL monthly (≈ 33.5 USD) to collect pufferfish using nets and some samples were also collected personally by the researchers by hook and line. Permission to collect pufferfish from specified fishers for scientific research purposes was granted from the Turkish Ministry of Agriculture and Forestry and General Directorate of Water Products. A total of 1360 pufferfish were sampled from this study, 216 from Finike, 817 from Fethiye (both Turkey), and 327 from Cyprus.

The total length (TL) and live (wet) weight (W) of all fish were measured to the nearest 0.1 cm and the nearest 0.1 g, respectively, while their gonads and livers were weighed to the nearest 0.01 g. The parameter of length-weight relationships (LWRs) of the form $W = a \cdot L^b$ were estimated through re-expression of the LWR equations in linearized form from the 817 Fethiye samples, i.e.,

$$\log(W) = \log(a) + b \cdot \log(L) \quad (1)$$

The von Bertalanffy growth function (VBGF), which is commonly used to describe the growth of fish has the form:

$$L_t = L_\infty \left(1 - e^{-K(t-t0)}\right) \quad (2)$$

where L_t is the length at age t, L_∞ is the asymptotic length, i.e., the mean length the individuals of a given population would reach if they grew indefinitely, K is the rate, of dimension time^{-1} (here: year^{-1}) at which L_∞ is approached, and t_0 is the age at zero length.

Here, a seasonally oscillating version of Equation (2) was used to analyse the available data which has the form:

$$L_t = L_\infty\{1 - \exp - [K(t - t_0) + S(t) - S(t_0)]\} \quad (3)$$

where $S(t) = (CK/2\pi) \cdot \sin(2\pi(t - t_s))$, $S(t_0) = (CK/2\pi) \cdot \sin(2\pi(t_0 - t_s))$, and L_∞, K and t_0 are defined as previously, but which has two additional parameters: C and t_s. Of these, the former is easiest to visualize, as it expresses the amplitude of the growth oscillations. When C = 0, Equation (3) simplifies back to Equation (2). When $C = 0.5$, the seasonal growth oscillations are such that growth rate increases by 50% at the peak of the 'growth season' (i.e., in 'summer'), and, briefly, declines by 50% in 'winter'. When $C = 1$, growth increases by 100%, doubling during 'summer', and becoming zero in the depth of 'winter'. The other new parameter, t_s expresses the time elapsed between $t = 0$ and the start of a sinusoid growth oscillation. However, visualization is facilitated if we define $t_s + 0.5$ = WP ('Winter Point'), which expresses, as a fraction of the year, the period when growth is slowest.

Note that longevity (t_{max}) can be approximated by $t_{max} \approx 3/K$, and that the mutual compatibility of the growth parameters L_∞ and K can be evaluated by the growth performance index

$$Ø' = \log(K) + 2\log(L_\infty) \quad (4)$$

which should be roughly similar between populations of the same species and between taxonomically related species of similar shapes (Pauly 1998).

The parameters L_{∞}, K, WP and C of Equation (3) were estimated through the ELEFAN method, which trace multiple growth curves through length-frequency (L/F) samples arranged in time. Each curve passes though peaks (represented by black, positive histograms, and deemed to represent age classes), and through the trough between peaks (represented by white, negative histograms). Peaks and troughs are identified by a simple high-pass filter, i.e., a running average which leads to definition of peaks as those parts of a length-frequency distribution that are above the corresponding running average and conversely for the troughs separating peaks. From a multiplicity of growth curves, each with a different set of growth parameters, the one is retained (along with the values of L_{∞}, K, WP and C which define it) which has the highest score in linking the peaks of L/F distributions, whose 'point' values are positive, while avoiding troughs, whose point values are negative (Pauly 1991, 1998). The software used here to implement the ELEFAN method was FiSAT, documented in Gayanilo et al. (2005).

Only Fethiye samples (Table 1) were used to estimate fecundity. The gonads were removed, weighed and preserved in a 10% buffered formaldehyde solution. To identify the reproductive season, temporal changes in the gonadosomatic index were assessed using the relation: GSI = 100·× (Wg/(W – Wg)) where Wg is the gonad weight and W is the live body weight. Also, the hepato-somatic index was computed as an indicator of reserves in the liver, i.e., HSI = 100·× (Wl/(W – Wl)) where Wl and W represent liver weight and live body weight, respectively. Finally, the overall plumpness of individuals was determined from their condition factor, or CF = $100 \cdot W/L^3$, where W is in g, and L in cm (TL).

The maturity stages of female samples were assigned to one of six stages based on macroscopic examination: immature, developing, developed, spawning capable/running, spent and resting (Murua et al. 2003). The mean length at first maturity/spawning (L_m) was estimated by plotting the fraction of mature individual females and males against their lengths, and fitting a logistic curve, with L_m being the length at which, in a given population, 50% of the individuals are mature and can be expected to spawn.

Also, the ratio $L_{max}{}^{D}/L_m{}^{D}$, was computed, where L_{max} is the maximum reported length in a population, L_m is a defined in the above paragraph and D = b(1 – d), with b being the exponent of a LWR, (and here set at 3; see below), and d is the exponent of a relationship between the gill surface area (S) and body weight (W) of the form $S \propto W^d$, with d set at 0.75, as befit a small fish (Pauly 1984).

For the spawning strategy investigation, we used the peak reproductive period based on increased mean GSI levels measured from the samples for the fecundity and histological sectioning which showed one distinct annual spawning season. Therefore, ovary samples were collected in April and May 2021. The oocyte size–frequency method was used for females with migratory nucleus or early hydrated oocytes to assess their fecundity; here, following Murua et al. (2003), the highly advanced oocytes (≥500 µm, as assessed using a Zeiss Labscope App [version 1.3.1] for iPad) were used for batch fecundity estimation, as the results become similar to the hydrated-oocyte method. Given these considerations, three subsamples, weighing between 20–40 mg, were taken from the anterior, middle and posterior parts of the ovaries.

Histological analyses were performed on 90 ovaries. Tissues were removed from the center of each ovary, fixed in 10% formalin solution, dehydrated in an increasingly strong series of ethanol solutions, then embedded in paraffin. Tissue sections of 5 µm, sliced with a microtome were stained with Mayer's hematoxylin and eosin, then mounted on a slide, then examined with an Olympus BX51 light microscope equipped with an Olympus DP72 digital camera (Roberts et al. 2012). The diameters of oocytes were validated by a second person using Leica image analysis software.

Stomach fullness was calculated on a subset of samples which included 465 fish sampled off Fethiye from December 2020 to May 2021 and 327 fish sampled off Cyprus from May 2020 to October 2021 (except for August and September 2020 due to COVID restrictions, compensated for by data for August and September 2021). Stomach fullness was measured using a 4-step scale with the first indicating an essentially empty stomach, the second stomachs that are less than half full, the third stomachs that are more than half full, and the fourth consisting of full stomachs. Next, a visual identification of items in the stomachs of 428 fish from Fethiye was performed using a Zeiss stereoscope; these items were identified to the lowest possible taxa, and then grouped as crustaceans, molluscs, gastropods, bivalves, echinoderms, fish, polychaetes, eggs, seagrass and sand. The number of items were counted in each stomach, if items were partially digested and could still be identified to species level or family or genus level, they were used, if none of those could be determined, then the sample was excluded from the results. If the specimen was identifiable, the following references were used to identify the species: Wirtz and Debelius 2003; Ballesteros and Llobet 2015, and asking expert colleagues. Visual identification of items in the stomachs of fish sampled off Cyprus were grouped into hermit crabs, crabs, barnacles, gastropods, fish, cephalopod ink and sand, but were not classified to species level.

A Wilcoxon Rank Sum test was used to determine if stomach fullness outside of the spawning season was different from that within the spawning season in Turkey (April-May, 2021 for Fethiye) and Cyprus (April-July 2020). A pairwise Permutational multivariate analysis of variance (PERMANOVA) was used to test whether the differences between sampling locations were statistically significant. For this, a matrix was prepared in the PRIMER software program using total length in cm (TL), weight in g (W), gonad weight in g (Wg), and liver weight in g (Wl) by assigning the location as a fixed factor (Clarke and Gorley 2001; Clarke and Gorley 2006), which was tested after the logx+1 transformation. A multivariate analysis in the form of a principal component analysis (PCA; Hotelling 1933), used to determine the ability of TL to distinguish among groups using the same data as the PERMANOVA analysis. The function princomp() with a correlation matrix was used to create a PCA after removing juvenile samples and samples that contained N/A values (R Core Team 2021).

Results

The number, sex and M/F ratio of *Torquigener hypselogeneion* used in this study are provided in Table 2. The length-frequency data by site are presented in Suppl. materials 1–3. Table 4 and Figure 3 prepresents LWRs for *T. hypselogeneion*. The LWR in Table 4 suggest that *T. hypselogeneion* males get thinner as they grow, whereas the females maintain the same body shape, as indicated by fact that the exponent (b) of their LWR is near 3, implying isometric growth.

Figure 4 presents the results of the analysis of the L/F data collected near Fethiye. Figure 4A suggests an asymptotic length for *T. hypselogeneion* (L_∞) of 17.4 cm (TL). The scan of K-values in Figure 4B suggest a best-fitting growth curve when

Table 2. Sex and Male / Female ratio of *Torquigener hypselogeneion*, by location.

Location	Finike	Fethiye	Cyprus
Juvenile	6	115	8
Female	109	505	155
Male	101	197	164
M / F	0.93 : 1	0.39 : 1	1.06 : 1

Table 3. *Torquigener hypsolegenion* individual GSI results and record details from this study.

Locality	Date	Sex	TL (cm)	TW (g)	GW (g)	GSI %
Fethiye	May.20	F	12.5	41.1	11.3	37.9
Cyprus	Jun.20	F	9.5	22.4	6.68	42.5

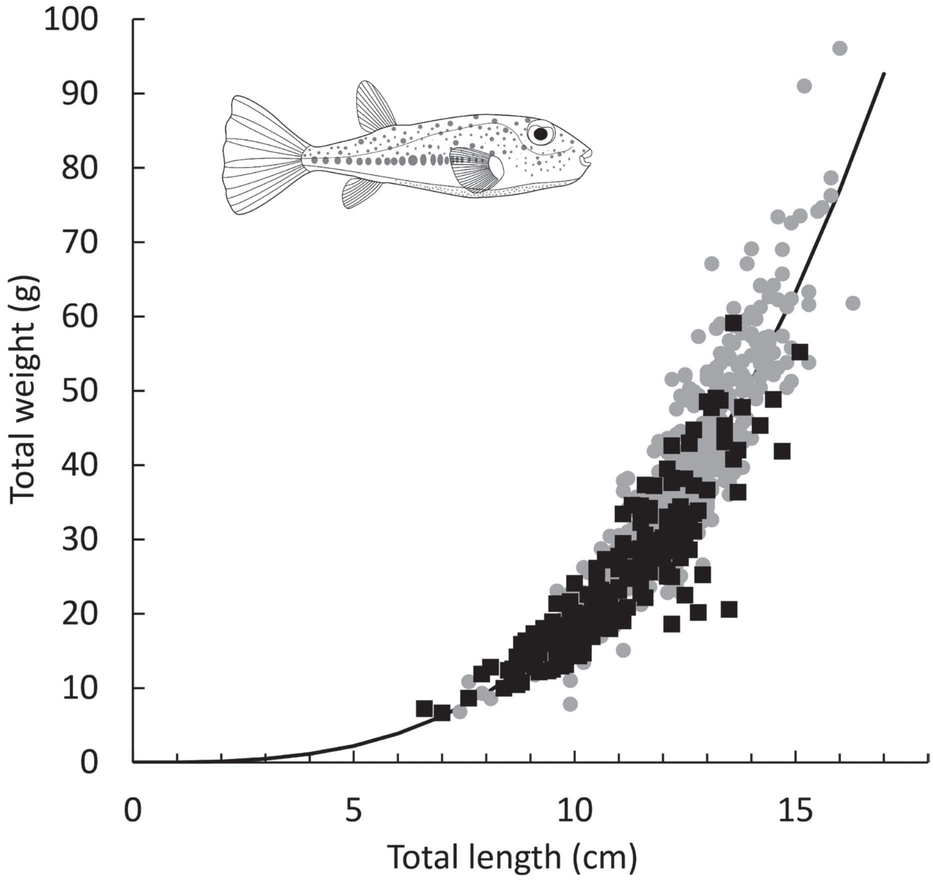

Figure 3. Illustrating the length-weight relationship (LWR, of the form $W = a{\cdot}L^b$) of *Torquigener hypselogeneion*, based on 817 individuals (sexes combined) sampled near Fethiye, with a = 0.0165 and b = 3.0471 and r^2 = 0.907; see also Table 3). Note that the females (dots) reach larger sizes than the males (squares).

K = 0.96 year^{-1} when WP = 0.2 and C = 3, and Figure 4C shows this best-fitting growth curve, superposed on the L/F data expressed in terms of peaks (black histograms) and through (white histograms).

Figure 5 shows the data and plots used to estimate the mean length at first maturity (L_m) of *T. hypselogeneion* sampled near Fethiye. This led to an estimation of L_m = 10 cm (TL) for both sexes. This estimate of L_m, combined with the maximum length observed in Fethiye, L_{max} = 16.3 cm (referring to a female), leads to a ratio $L_{max}{}^D/L_m{}^D$ of 1.44.

Some specimens had very high GSI values. Thus, from Fethiye, there were ten fish with GSI ranging from 30–38%. From Cyprus, one specimen had a GSI of 42% (Table 3). The mean monthly GSI of fish sampled off Fethiye during the peak of the 2020 spawning season were more than double than those found in the spawning season one year later (15% and 17% respectively for April and May 2020 as shown in Figure 6 compared to 6.5%, 8% and 11% for April, May and June 2021, 2021 not shown in Figure 6).

The condition factor gradually declined after the onset of the spawning seasons in Fethiye, but Finike samples showed only a very slight dip. In Northern Cyprus, the condition factor peaked in June right after the start of the peak spawning season.

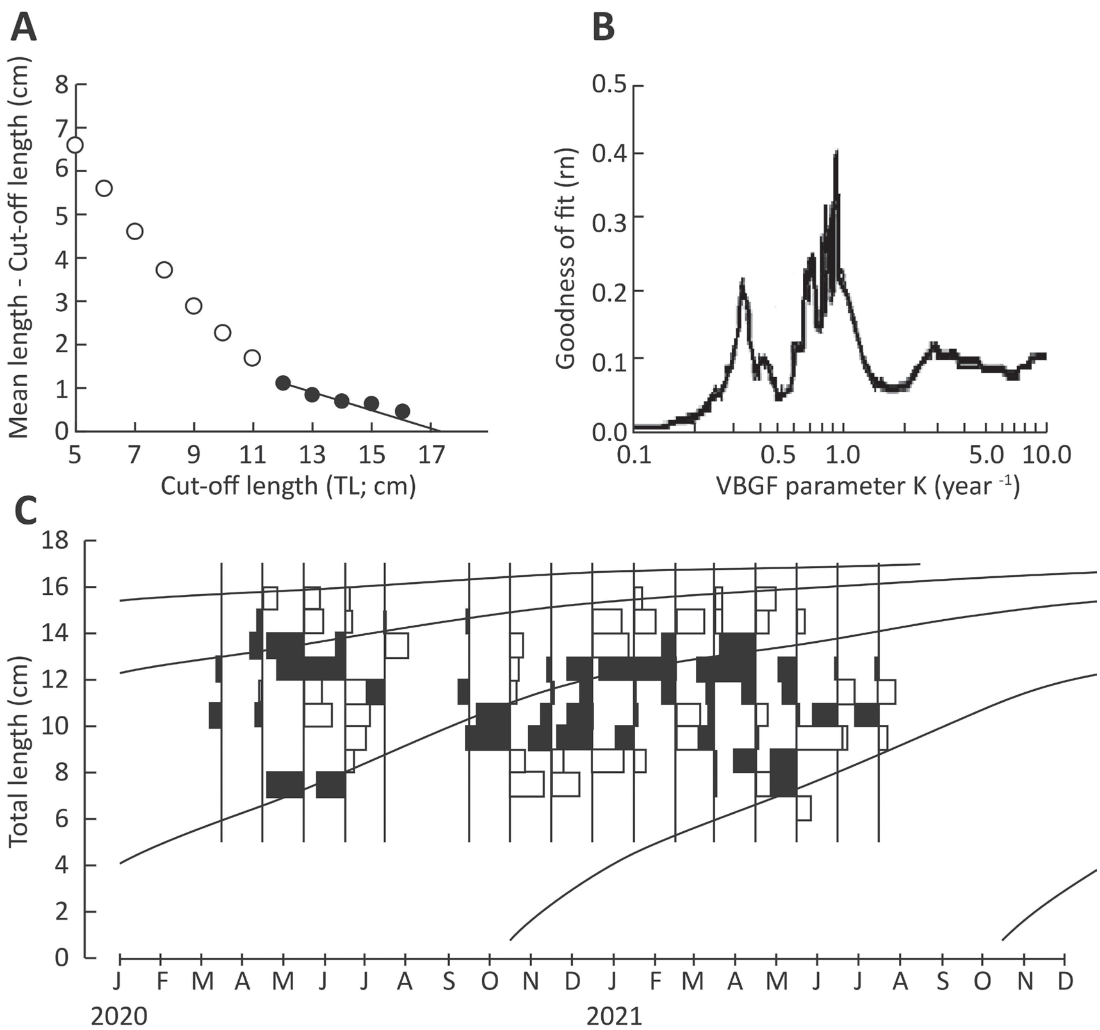

Figure 4. ELEFAN analyses of the length-frequency data of *Torquigener hypselogeneion* in Suppl. material 2. **A:** Preliminary estimation of L_∞ = 17.4 cm using the Wetherall Plot routine of the FiSAT software. **B:** Goodness of fit for a range of K values (and L_∞ = 17.4 cm, with C = 0.3 and WP = 0.2), showing that K = 0.96 year^{-1} provides the best fit. **C:** Growth curve defined by L_∞ = 17.4, cm, K = 0.96 year^{-1}, C = 0.3 and WP = 0.2) superposed onto the restructured L/F data in Suppl. material 2, i.e., with 'peaks' re-expressed as black (positive) and troughs as white (negative) histograms.

According to the PERMANOVA results: total length, total weight, gonad weight, and liver weight were significantly different by means of locations (Table 5).

The first and second principal component axes (PC1 and PC2) explained about 76% and 14% of the variation among the length and weight variables, respectively. Positive regressions for all four variables (length and body weight, gonad liver weight) were correlated with PC1, whereas PC2 negatively correlated with gonad weight and strongly correlated with length (Table 6).

Table 4. Length-weight relationship parameters for males, females and both sexes pooled for *T. hypselogeneion* based on Fethiye samples.

	N	a	b	r^2	95% Confidence Interval of b
Male	197	0.0407	2.6543	0.834	2.487–2.821
Female	505	0.0153	3.0869	0.854	2.975–3.199
Pooled	817	0.0165	3.0471	0.907	2.938–3.115

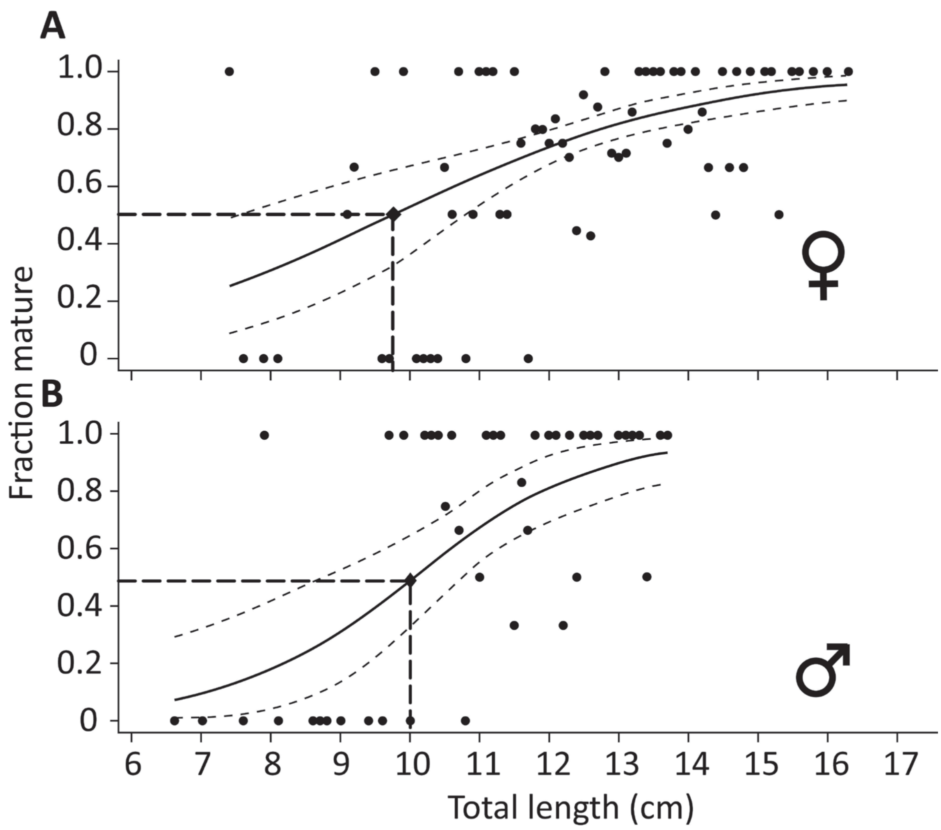

Figure 5. Length at first maturity (L_m) of *Torquigener hypselogeneion* collected from Fethiye in 2020 and 2021, with the data points fitted logistic curves whose 95% confidence intervals (dotted lines) are also shown, along with the lengths at which the probability of being mature is 0.5. **A:** Female, with L_m = 9.8 cm. **B:** Males, with L_m = 10.0 cm. The difference between these two estimates of mean length at first maturity is not significant and $L_m \approx$ 10.0 cm (TL) for both sexes.

Table 5. The pairwise Permutational multivariate analysis of variance (Permanova) results compared for the study areas.

Pairs	Degrees of freedom	p value	Pseudo-F statistics
Fethiye-Cyprus	897	0.001	87.240
Fethiye-Finike	791	0.001	52.410
Cyprus-Finike	368	0.008	26.963

Table 6. Variance in 4 traits of *Torquigener hypselogeneion* sampled at 3 sites explained by the first two axes of a Principle Component Analysis (PCA).

Variable	PC1 (75.7%)	PC2 (13.8%)
Total length	0.501	0.584
Body weight	0.552	0.234
Liver weight	0.492	-0.165
Gonad weight	0.449	-0.760

The PCA result shows that adult fish from Finike (N = 210) and Cyprus (N = 318) do not appear to differ, whereas the fish sampled in Fethiye (N = 678) showed a greater variance along both axes (Figure 7). This indicates that fish from Fethiye have a range of size and weights overlapping with samples from Finike and Cyprus, although only samples from Fethiye contained the heaviest and largest fish.

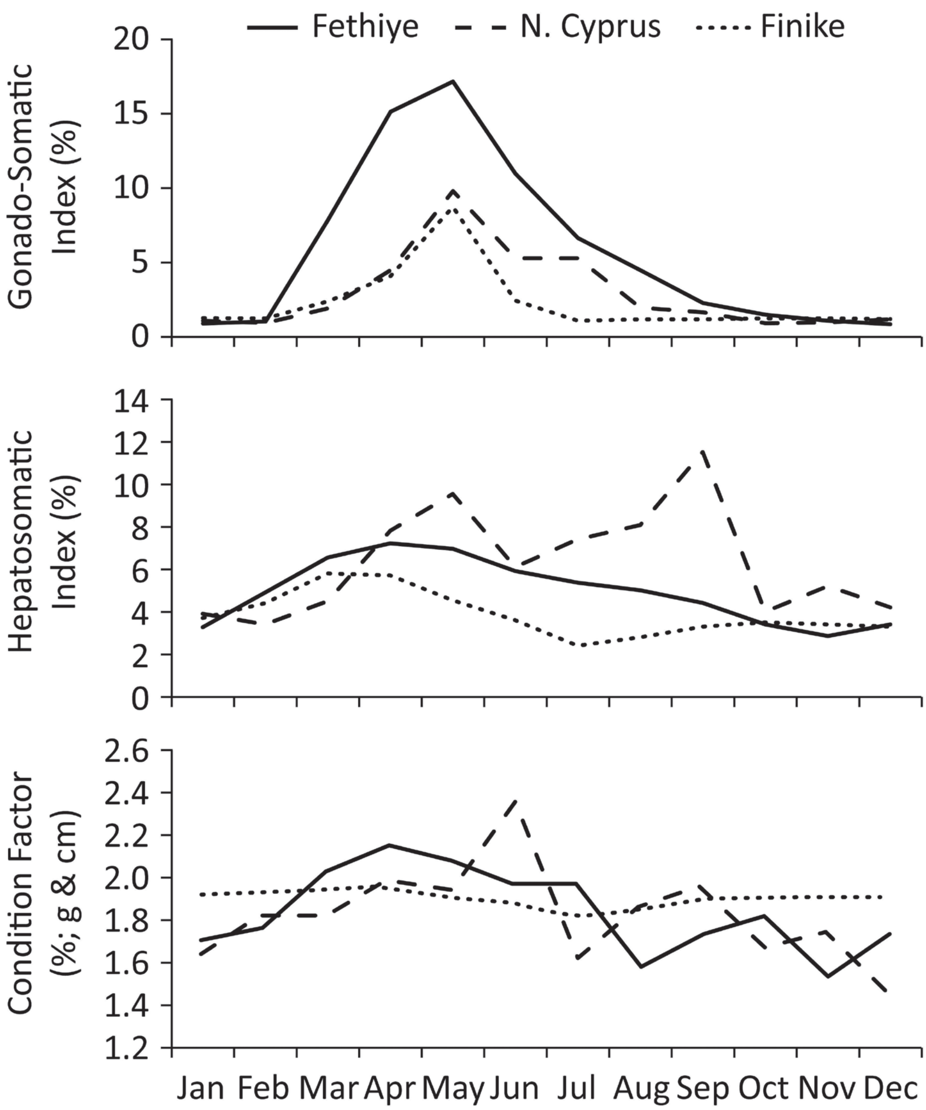

Figure 6. A) Mean monthly Gonado-Somatic Index; B) Hepato-Somatic Index; and C) Condition Factor for Fethiye (2020), Northern Cyprus (2020), and Finike (2017).

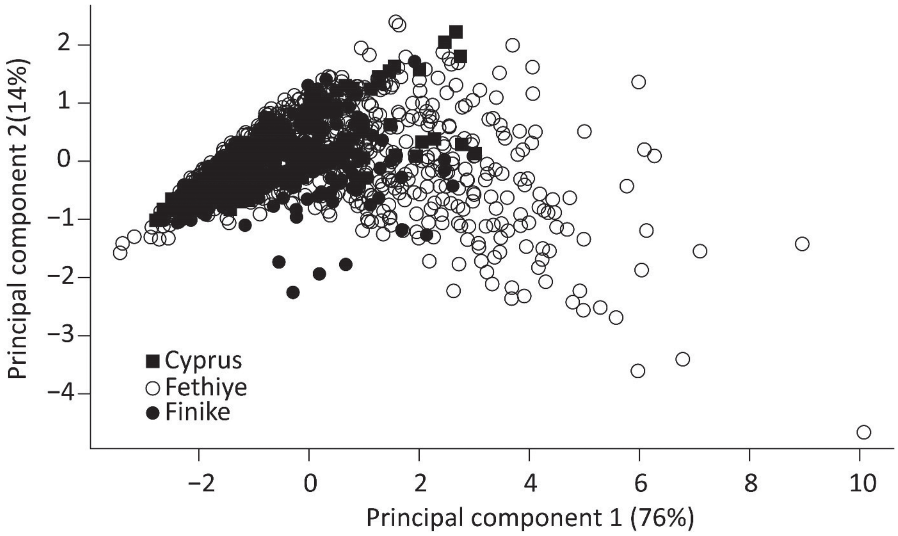

Figure 7. Principal component analysis of four variables (length and body weight, gonad, liver weight) in *Torquigener hypselogeneion* samples collected in Fethiye and Finike (Turkey) and Cyprus. Note overlap of Finike and Cyprus samples.

The ovaries of *T. hypselogeneion* appear to be organized into synchronous groups, i.e., they show two distinct sized group of ovaries, but fecundity appears to be determinate. Primary growth (PG) oocytes and vitellogenic oocytes (Vit) were clearly recognized during the spawning period (Figure 8). Atresia was very commonly observed in female ovaries during both the previtellogenesis and vitellogenesis phases. Generally, hydrated oocytes (H) and post ovulatory follicles (POF) are observed rarely in samplings (van Damme 2010); still, both stages were observed in our samples during May. Spawning pattern was evaluated as batch spawner due to distinct hiatus between oocytes groups in each development phase of ovaries and the occurrence of POF during months for which samples were available. Thus, *T. hypselogeneion* spawns as successive batches of hydrated eggs at intervals of several days after a pool of yolked oocytes (group-synchronous development) enter final maturation in clutches. Based on 15 female ovaries measured from during peak spawning period (see Figure 6), vitellogenic oocytes ranged from 240–420 µm in diameter.

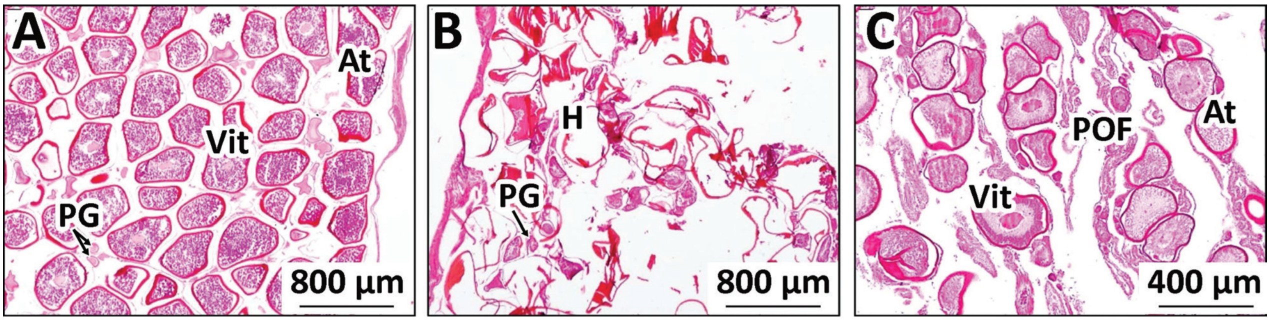

Figure 8. Stages of oocyte development in *Torquigener hypselogeneion*. Histological sections show **A:** primary growth oocytes (PG), vitellogenic oocytes (Vit) and atresia (At); **B:** primary growth oocytes (PG) and hydrated oocytes (H); and **C:** vitellogenic oocytes (Vit), post ovulatory follicles (POF) and atresia (At). Scale bars 800 µm (**A, B**); 400 µm (**C**).

The fecundity study was conducted on females with ripe ovaries ranging from 11.8 to 15.8 cm in total length and 31.6 and 78.7 g in weight during the peak spawning season (mostly from late April, 2021). Late development phase of oocytes (late vitellogenesis) was examined under the microscope. Fecundity was found to be between 448 to 3,165 eggs per gram body weight and the fecundity of an average-sized female (13 cm TL and 48 g in W) *T. hypselogeneion* was found to be 1,250 eggs per gram body weight. No relationship could be established between fecundity and fish size.

Table 7 shows the stomach fullness of specimens of *T. hypselogeneion* from Fethiye and Cyprus. There was a significant ($P < 0.001$) reduction in stomach content fullness during the spawning period in April and May from Fethiye (Figure 9B), compared to outside the spawning season (December to March). However, in Cyprus, the relationship was not significant, but empty stomachs were prominent at the end of the spawning season in July, and perhaps in August and September, if to a lesser extent (Figure 9A).

Table 7. Overall stomach fullness (SF) in %, as evaluated on a 4-point scale, i.e., 'Empty' (0), 'Up to ½ full' (0< - <0.5), 'More than ½ full' (≥ ½ - <1) and 'Full' (1), of specimens of *Torquigener hypselogeneion* from Fethiye and Cyprus.

Stomach Fullness	N	0	0< - <0.5	≥ 1/2 - <1	1
Fethiye	465	21	36	29	14
Cyprus	327	38	25	18	19

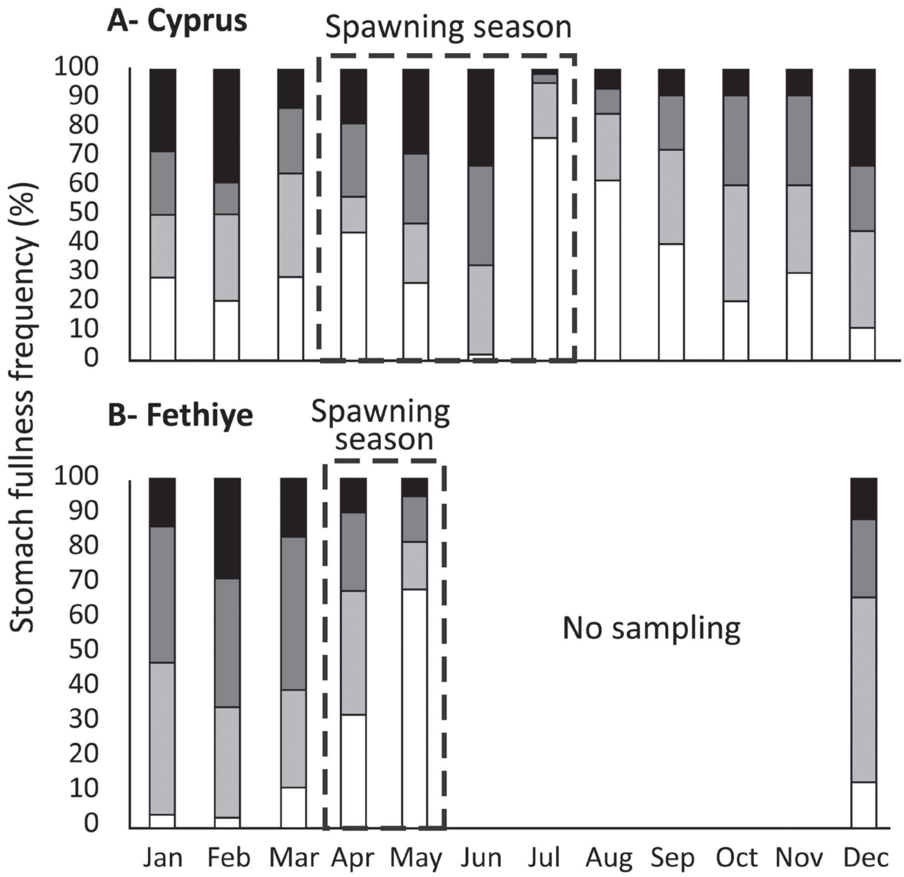

Figure 9. Comparison of stomach fullness ratio frequency for Cyprus (**A**) and Fethiye (**B**) White = empty stomachs; light grey: stomachs less than ½ full; darker grey: stomachs half full or more; black: full stomachs.

In Fethiye samples, small gastropods made up much of the diet, with the invasive *Cerithim scabridum* Philippi, 1848 (10–25 mm in length), along with similar-sized gastropods such as *Bittium reticulatum* (da Costa, 1778), *Phorchus turbinatus* (Born, 1778) being found in 46% of specimens, followed by crustaceans with 32%, which mainly included hermit crabs using gastropod shells as their shelter, crabs (mainly juvenile *Calappa granulata* (Linnaeus, 1767)), barnacles (with entire shells), and squat lobsters (*Galathea squamifera* Leach, 1814). The third most frequent prey group was sea urchins. Here, both native species were identified (*Arbacia lixula* (Linnaeus, 1758) and *Paracentrotus lividus* (Lamarck, 1816). Bivalves were found in 7% of the stomachs and included juvenile forms of *Chamelea gallina* (Linnaeus, 1758), *Clausinella fasciata* (da Costa, 1758), *Arculata senhousia* (Benson, 1842). Other items in 1–3% of stomachs were *Polititapes aureus* (Gmelin, 1791), seagrass, small fish and fish eggs, polychaetes and cephalopods, via their long-lasting ink. In Cyprus, the stomach contents consisted mainly of crustaceans, with crabs contributing 44%, hermit crabs 23% and barnacles 17%, followed by cephalopod ink 9%, and gastropods and fish contributing the rest.

Discussion

This study provides new information on its biological characteristics such as growth, spawning season, reproduction and diet for *T. hypselogeneion*, the tiny but deadly invasive pufferfish in the Mediterranean. In comparing three different populations, features are emphasized which pertain to the Fethiye specimens, where this species apparently first established itself in the Mediterranean (Bilecenoğlu 2003, 2005).

The maximum length sampled in Fethiye was 16.3 cm (TL), very close to the 16.5 cm maximum length reported by Kebapçioğlu and Beğburs (2017) from Finike Bay, Turkey, both compatible with the asymptotic length found here of 17.4 cm. This makes the 18.5 cm record from Israel (Golani et al. 2021) a case of gigantism, as reported, if at a much larger scale, for another invasive in in the Eastern Mediterranean, *Lagocephalus sceleratus* (Ulman et al. 2022). Invasive species have frequently been found to grow larger in their invaded environments than their native ones (Darling et al. 2011; Ulman et al. 2022), suggesting that their resources and conditions (including lower numbers of competitors and potential predators) in the region they have invaded are superior to those of their native regions. Also note that *T. hypselogeneion* is limited to smaller maximum lengths along the coast of Cyprus than in the bays of Finike and Fethiye (see Suppl. materials 1–3), likely due to the higher temperature in the waters of Cyprus (Pauly 2021a). Table 8 suggests that the growth performance of *T. hypselogeneion* is low, albeit not extremely so, when compared to other pufferfishes. The PCA analysis showed that the Fethiye site was only significant from that of both Finike and Cyprus, whereas the PERMANOVA results showed that the three sites were statistically different in maximum lengths, which makes sense as PCA shows the key features of the data, whereas PERMANOVA shows the differences between the factors.

Table 8. Comparison of the growth performance of *Torquigener hypselogeneion* with that of other pufferfish species using $\phi' = \log(K)+2\log(L_\infty)$.

Species	L_∞ (cm)	K ($year^{-1}$)	ϕ'	Reference
Lagocephalus sceleratus ♀&♂	82.0	0.5	3.52	Michailidis (2010)
	88.7	0.27	3.43	Ulman et al. (2021)
	81.1	0.26	3.23	Sabrah et al. (2006)
Sphoeroides maculatus ♀	28.2	0.607	2.68	Laroche and Davis (1973)
Sphoeroides maculatus ♂	24.5	0.620	2.57	Laroche and Davis (1973)
Sphoeroides testudineus ♀&♂	30.0	0.51	2.66	Pauly (1991)
T. hypselogeneion ♀&♂	17.4	0.96	2.46	**This study**
Contusus richei ♀	18.9	0.326	2.07	Pauly (1991)
Contusus richei ♂	12.5	0.362	1.77	Pauly (1991)

A very low male to female ratio (M:F = 0.39:1) was found for all months Fethiye, whereas Finike had a ratio of 0.93:1, and Cyprus had a ratio of 1.06:1. Another recent study from Cyprus reported a very high male to female ratio close to 4:1 (Chartosia et al. 2021). The stronger female ratio from Fethiye may be due to our samples having been obtained from deeper waters (down to 75 m), given that Mutlu et al. (2021) reported higher female abundances with increasing depth. Ontogenetic shifts also occur in niche habitats, as juveniles were commonly seen in shallower habitats at depths from 3–8 m in Kidrak Beach, Fethiye, Turkey in 2020 and 2021, while the adults generally occur in deeper waters (AU, pers. obs.). The multivariate analysis showed Fethiye fish to also be larger and heavier. Since Fethiye was the first locality where this species seems to have established itself in the Mediterranean, it can be assumed that the conditions are favorable for them here, possibly relating to food availability.

Torquigener hypselogeneion appears to reach maturity at 1 year, when its L_{max}^D/L_m^D ratio is about 1.44, which is well within the 95% confidence interval (1.22–1.53) of the 1.35 threshold value shown to trigger first maturation and spawning in bony fishes (Chen et al. 2021; Pauly 2021a, 2021b).

Of our three sites, the spawning season was longest in Fethiye (lasting from March to August in 2020), then Cyprus (from March to July), while it lasted only two months in Finike (April to May). These three sites are at about the same latitude, but the mean water temperature is slightly higher in the more eastward Cyprus. This somewhat aligns with the results on an extensive study by Tsikliras et al. (2010) who found the spawning seasons of numerous fish species to be longer in the Western than in the Eastern Mediterranean. The majority of Mediterranean stocks spawn in late spring/early summer, coinciding with the high zooplankton abundances in coastal waters (Fernandez de Puelles 2003) that follow early spring phytoplankton blooms (Gaudy and Champalbert 1998).

An extensive study on GSI values for Mediterranean fish species that analyzed 237 stocks belonging to 81 species reported a Mediterranean mean GSI value of 6.8% (in spawning season only) for both sexes, 8.6% for females and a significantly lower rate of 4.2% for males (Tsikliras et al. 2010). Thus, our finding of the April and May 2020 mean GSI values (both sexes combined) from Fethiye of 16% and 17% are much higher than the Mediterranean average, and are even higher when only females are considered (17% and 19%). We also report the highest individual GSI rates recorded so for Mediterranean fish (Table 3).

For Fethiye and Finike, as expected, the peak hepato-somatic index (HSI) occurred one month before the peak spawning period, and then a gradual decrease in HSI was observed as energy reserves were used for gonadal development, as found in other studies (Ramadan and El-Halfawy 2019). In Cyprus a similar smaller peak in HSI also occurred just before the spawning season in April, but a stronger peak occurred in August and September suggesting that the fish of this population store a higher percentage of energy in their livers than the other two populations. The condition factor, which describes the plumpness of individuals declined following the spawning period in Fethiye and Cyprus, but remained relatively stable from Finike samples, which perhaps suggest that this population has fewer food resources, and that therefore, individual weights fluctuate less.

The stark differences in the spawning periods between our three sites is intriguing. Based on GSI values, an extended period was found in Fethiye. However, we performed fecundity studies only in late April, and histological studies in May, i.e., both during peak spawning season. Because there was another slight peak in GSI values during autumn period, total fecundity per annum per female was not estimated. Additionally, we could not evaluate whether this species continued to spawn, or whether the ovaries transform to atretic stages. Future studies should cover both the spawning and non-spawning period to better understand its adaptation mechanisms considering any possible changes to its reproductive strategies, as well as total fecundity of this species for determining annual egg production.

Another intriguing result was that the fecundity of *T. hypselogeneion* did not vary with size, i.e., small individuals contained as many eggs as large ones. One explanation for this may be found in the morphological adaptations which allow pufferfish to puff, such as their stretchable skin and lack of pleural ribs and pelvis. Possibly, this allows for an extra space for the storage of eggs that may be (between lengths of 10 to 16 cm), independent of body size. This would also explain why, at least in Fethiye, feeding is reduced during the spawning season, which would also allow the abdominal cavity to accommodate more eggs. Oocyte sizes during vitellogenesis are very small (avg. 350 μm in diameter), which allows for a high fecundity. During hydration, egg size may strictly increase (Nissling et al. 2017); however, no information is available on the shape, diameter and pigmentation of *T. hypselogeneion* eggs in the natural environment.

While its extremely powerful regenerative fused teeth (Thiery et al. 2017) provide pufferfishes access to tough-shelled animals, such as barnacles, these fishes, especially the small species, also use jets of water out of the mouth to access small benthic animals buried in the sandy seafloor; indeed, their ability to puff may have evolved to amplify this behavior (Wainwright and Turingan 1997). Thus, it is not surprising that the stomach contents of *T. hypselogeneion* consists predominantly of very small gastropods such as *Cerithium scabridum*, *Bittium reticulatum* and *Trochus turbinatus*. Interestingly, it is clear from the stomach content analysis that *T. flavimaculous* is able to digest the hard shells of barnacles, gastropods, crabs and other molluscs as most were found in various stages of digestion. This ability to digest both tough and soft items surely expands its niche helping to make it a more successful predator. They may be controlling populations of the invasive *Cerithium scabridum*, however due to the gastropods miniscule size, it is doubtful that this would offer any benefits to the ecosystem in general, as *C. scabridum* is very highly abundant in the region. The only predator found so far for *T. hypselogeneion* is the larger invasive pufferfish in the region, *Lagocephalus sceleratus* (Ulman et al. 2021), which makes sense as these are the two pufferfish species with the highest TTX poison, and thus would be able to handle these high levels of poison with no risk of fatality.

In the waters off of Cyprus, the native sea urchin populations of *P. lividus* started declining around 2010 and collapsed in 2014 (Çiçek 2019). A study of *T. flavimaculosus* diet from Cyprus (Chartosia et al. 2021) revealed sea urchins as a major prey item, also suggesting that predation from *T. hypselogeneion* may have attributed to the collapse of *P. lividus* in the Eastern Mediterranean region (Chartosia et al. 2021). However, another study links the sea urchin decline to ocean warming (Yeruham et al. 2015), so the culprit is not clear. Five specimens of *T. hypselogeneion* caught on February 1st 2021 from Fethiye had from 7 to 25 hermit crabs in their stomachs, which however, did not have their usual gastropod shells. This suggests that *T. hypselogeneion* learned to suck the hermit crab out of their shells.

From *in situ* observations of *T. hypselogeneion* in their environments in both Turkey and Cyprus, aggressive and competitive feeding behaviour was noticed on a few occasions in the presence of blood from spearfishing lionfish and dead crabs from cleaning lost fishing nets. Chartosia et al. (2021) classified *T. hypselogeneion* as an omnivore, as chunks of plants were found in seven out of 104 stomachs (i.e., 6.7%); in this study, plant material occurred in <3% of stomachs, which may have been consumed with live animals attached to them. In the region, several types of algae generally begin to grow in spring for a few months and then disappear for the remainder of the year due to heavy predation from Siganidae (invasive rabbitfishes) (Sala et. al. 2011), and the spring season was sampled here; the area also has year-round populations of *Posidonia oceanica* seagrass that overlap with *T. hypselogeneion* habitat. However, there are many species of algae, some of which peak in autumn or winter (Turna et al. 2002), which was not captured here, which is a caveat of this study. Thus, it may be more appropriate to view *T. hypselogeneion* as a generalist carnivore with a high preference for benthic invertebrates.

Conclusion

Marine invasive species research is highly interesting in the Eastern Mediterranean, as the marine ecosystems are undergoing a major transition caused by the influx of alien species. Hence some of their adaptations may differ at separate locations based on differences in the ecosystem structure or abiotic variables. The three *Torquigener hypselogeneion* [conspecific *Torquigener flavimaculosus*] populations examined here showed some considerable variabilities with respect to sex ratios, spawning periods and diet compositions. Fethiye, where they were first noticed in high abundances in the Mediterranean seems to have favourable conditions for its growth and reproduction, with the highest GSI, and fecundity estimated for the first time. Our results are comparable with those for other small pufferfish; maturity is reached after one year, and longevity is about 4 years; the males become thinner as they grow, while the females maintain the same body shape. Fecundity is high, and largely size independent. The food mainly consists of small benthic animals, either chomped by powerful fused teeth, or likely blown of sandy seafloor be jets of waters, two adaptations providing access to a wide range of resources. Based on its high invasiveness and negative impacts to ecology of the Eastern Mediterranean and the human heath, we suggest that *T. hypselogeneion* be listed as a priority invasive species and that its population and impacts be closely monitored within the Mediterranean Sea.

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Ethics and permits

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Author contributions

AU- Conceptualization; AU, HE, CEO, BAC, HDA- Sample Design & Methodology; AU, BAC, HDA, HE, OC, ND, AL, SM- Investigation; AU, EO, CEO, BAC, HAD- Resources; TY, ND, DP, EC- Software; AU, HE, CEO, SM, BAC, HDA- Validation; AU, ND, CEO, HE, DP, EC- Formal analysis; AU, TY- Data Curation; AU, ND- Writing - Original draft; AU, ND, DP- Writing - Review and Editing; AU, TY, ND, DP, EC, AL-Visualization.

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Supplementary material 1

Length-frequency data of *Torquigener hypselogeneion* collected in Finike, Turkey from March 2017 to February 2018

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Data type: table (docx. file)

Link: https://doi.org/10.3391/ai.2023.18.1.103438.suppl1

Supplementary material 2

Length-frequency data of *Torquigener hypselogeneion* collected in Fethiye from March 2020 to August 2021

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Data type: table (docx. file)
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Link: https://doi.org/10.3391/ai.2023.18.1.103438.suppl2

Supplementary material 3

Length-frequency data of *Torquigener hypselogeneion* collected in Cyprus from May 2020 to April 2021 with additional data for August and September 2021

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Supplementary material 4

Georeferenced coordinates for study sites and first records provided in Fig. 2

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