Geographic range expansion of *Ephippion guttifer* (Tetraodontidae) in the north-eastern Atlantic

R. BAÑÓN[1], A. ALONSO-FERNÁNDEZ[1,2], D. BARROS-GARCÍA[3], M. B. RIOS[4] AND A. DE CARLOS[3]

[1]Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas, IIM-CSIC, c/ Eduardo Cabello 6, 36208 Vigo, Pontevedra, Spain

[2]Asociation Ecoloxía Azul – Blue Ecology. c/Herreros 28, 36002 Pontevedra, Spain

[3]Departamento de Bioquímica, Xenética e Inmunoloxía, Facultade de Bioloxía, Universidade de Vigo, Rúa Fonte das Abelleiras s/n, 36310 Vigo, Spain

[4]Cofradía de Pescadores San José, Avda. de Bueu s/n 36940, Cangas do Morrazo, Pontevedra, Spain

Correspondence

R. BAÑÓN, Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Científicas, IIM-CSIC, c/ Eduardo Cabello 6, 36208 Vigo, Pontevedra, Spain

Email: anoplogaster@yahoo.es

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The first record of the prickly puffer *Ephippion guttifer* (Tetraodontidae) from Galician waters (north-west Spain) is reported based on a male specimen of 570 mm total length (L_T) caught in the Ría de Vigo. Morphometric, meristic and DNA barcode data confirmed the identification. Histological examination of reproductive tissue was carried out in this species for the first time, showing a mature male in an actively spawning phase. A historical revision invalidates a previous record and establishes this as the northernmost confirmed capture ever reported in the north-eastern Atlantic Ocean.

KEYWORDS

Distribution, NE Atlantic, reproductive biology, smooth puffer, tropicalization

There are 196 described species in 26 genera of tetraodontid puffer fishes (family Tetraodontidae), also commonly called blowfishes and globefishes, that live worldwide in tropical and temperate seas (Nelson *et al*., 2016). Tetraodontids are mostly marine fishes, but approximately 30 species spend their entire life cycles in freshwater, in tropical regions of South America, Central Africa and South-east Asia (Yamanoue *et al*., 2011). Most puffer fishes occur in shallow waters, but some species can be found at greater depths, such as the blunthead puffer *Sphoeroides pachygaster* (Müller & Troschel 1848), which may be found as deep as 480 m (Shipp, 1974).

Six species of tetraodontids have been recorded in Atlantic European coastal waters (Quéro *et al*., 2003; Bañón & Santás, 2011): the Caribbean sharpnose-puffer *Canthigaster rostrata* (Bloch 1786), the prickly puffer *Ephippion guttifer* (Bennett 1831), the oceanic puffer *Lagocephalus lagocephalus* (L. 1758), the smooth puffer *Lagocephalus laevigatus* (L. 1766), the Guinean puffer *Sphoeroides marmoratus* (Lowe 1838) and the blunthead puffer *S. pachygaster*.

Ephippion guttifer is a marine demersal fish inhabiting shallow coastal and estuarine environments to depths of *c*. 50 m (Shipp, 1974) and is associated with a variety of habitats including hard and muddy substrata (Shao *et al*., 2014). It is known in the eastern Atlantic Ocean from Morocco and the extreme western Mediterranean Sea southward along the entire west coast of Africa to Angola, near Benguela (Shao *et al*., 2014). There is one record from the Bay of Biscay (Tortonese, 1986) and it has also been reported off Portugal (Carneiro *et al*., 2014).

On 10 January 2017, a live specimen of *E. guttifer* (Figure 1) was caught in the trammel nets of local fishermen off Praia de Barra (41° 42' 46.021" N; 8° 51' 24.998" W) at a depth of *c*. 5 m in the mouth of the Ría de Vigo, in the southern part of Galicia, Spain. A dot distribution map was created based on georeferenced data reported in online marine biogeographic databases (OBIS and GBIF) and was completed with bibliographic records, mainly from the Mediterranean Sea (Hachaichi, 1981) and the Iberian Peninsula (Tortonese, 1986), including the present record (Figure 2). The fresh specimen of *E. guttifer* measured a total length (L_{T}) of 570 mm and additional morphometric and meristic data were collected from it following Shipp (1974). The specimen was then preserved in 70% ethanol and was deposited in the fish collection of the Museo Luis Iglesias de Ciencias Naturais in Santiago de Compostela with the reference number MHNUSC25103.

The body was elongated and inflatable. Two nostrils were located approximately halfway between the snout and anterior margin of the eye, one on each side of snout, each with two flaps. There were no scales, although small spinules were present on the belly near the anus. The dorsal and lateral surfaces of the body were greenish in colour, while the ventral surface was white. Numerous white spots covered the body over its pigmented area and caudal fin, changing their shape from elongate laterally to round posteriorly. The main morphometric and meristic data are given in Table I.

The structure of the nostrils, number of dorsal-fin rays and coloration pattern are diagnostic features that diagnose this specimen as *E. guttifer*. Measurements and meristic counts are in general agreement with previous descriptions of the species (Shipp, 1974; Matsuura, 2016). Minor differences could be attributed to the larger size of the sampled specimen with respect to specimens examined by Shipp (1974).

A muscle sample was collected from which to extract DNA and sequence the standard 5´ barcoding region of the mitochondrial *coI* gene, using the primer cocktail C_FishF1t1-C_FishR1t1 (Ivanova *et al*., 2007), following procedures described elsewhere (Bañón *et al*., 2016). A 651 nucleotide-long sequence was deposited in GenBank under accession number KY498013. The taxonomic identification of the sample was further explored by conducting a neighbour-joining (NJ) analysis using MEGA 6 (Tamura *et al*., 2013). Molecular, morphological and distributional data of this specimen can be found in the Barcode of Life Database (www.boldsystems.org) by searching for the project "Tetraodontiformes from Galician waters".

A NJ tree clustered in a strongly supported clade the *coI* sequence of the Galician specimen together with the only vouchered sequence of *E. guttifer* available in public databases (KP998097). Genetic distance between the *coI* sequences was 0.31%, while distances between the Galician specimen and other tetraodontid species available in the database varied between 13.21% from species of *Arothron* Müller 1841 to 17.59% from species of *Lagocephalus* Swainson 1839.

The morphological diagnosis of the species was corroborated by the molecular analysis. The *coI* cluster analysis also assigned the Galician sample to *E. guttifer*, showing a within-species genetic divergence in the range of mean values found for fishes (Ward *et al*., 2009). Comparison of nucleotide differences between *E. guttifer* and other tetraodontid sequences resulted in distance values like those previously documented among fish genera (Ward *et al*., 2009). DNA barcoding succeded in validating the identification of *E. guttifer*, as has also occurred in other tetraodontid taxa (Kaleshkumar *et al*., 2015).

Studies on reproductive biology are essential to understand fish population dynamics (Brown-Peterson *et al*., 2011). Marine pufferfishes have a range of annual reproductive cycles, from seasonal, with one or two actively reproductive periods to year-round gamete production (Valdez-Pineda *et al*., 2014; Sánchez-Cárdenas *et al*., 2011). However, information the reproductive biology of *E. guttifer* has not previously been reported.

Gonads were removed from the Galician specimen and immediately fixed in 3.6% buffered formalin. Serial subsamples of the gonad (five in total) were extracted, dehydrated, embedded in paraffin wax, sectioned at 3 μm and stained with haematoxylin and eosin for histological examination. The specimen was sexed and classified according to its corresponding maturity phase using histological criteria (Grier, 1981; Brown-Peterson *et al.*, 2011).

The Galician specimen was a male and its testes represented 1.3% of eviscerated mass. Testicular tissue was of the lobular type (Takashima & Hibiya, 1995), with spermatogonia development evident along the germinal epithelium (Figure 3(a),(b)) equivalent to the unrestricted spermatogonial type proposed by Grier (1981). The specimen's stage of maturity was established as the spawning capable phase (Brown-Peterson *et al.*, 2011), in the actively spawning subphase, since the testes exhibited spermatozoa in the lumen of the lobules (Figure 3(b)). Spermatogenesis is cystic in *E. guttifer*, with developing germ cells enclosed within germinal cysts formed by enveloping Sertoli-cell processes. Within each cyst maturation of germ cells is synchronous (Figure 3(c),(d)).

The most important result obtained from analyses of the gonads was that the specimen was sexually mature and potentially able to reproduce, since the testes exhibited spermatozoa in the lumen of the lobules (Brown-Peterson et al., 2011). Common in members of the Tetraodontidae family, *E. guttifer* seems to be a gonochoric species since no evidence of hermaphroditic tissue was found in any of the sequential histological slides examined. Although this is the first histological analysis of reproductive traits in *E. guttifer*, more specimens would be needed to robustly determine development stage of the gonads.

It has been reported in the literature that *E. guttifer* is an Atlantic African species with sporadic intrusions into the western Mediterranean Sea (Hachaichi, 1981). Its occurrence in Atlantic European waters is poorly documented and there are only two previous reports (Tortonese, 1986; Carneiro *et al*., 2014). However, these reports provided neither the exact catch location nor the morphological and descriptive characters needed to reliably identify *E. guttifer*. Tortonese (1986) reported one specimen from the Cantabrian Sea, off the Spanish coast of the Gulf of Biscay but the exact location was not stated. According to his previous work (Tortonese, 1973), the original record was reported by Dieuzeide (1955: 356), who only noted the same information in French '*signalé dans le Golfe de Gascogne*' (recorded in the Bay of Biscay). However, at that time, the only tetraodontid reported in the Spanish area of the Gulf of Biscay was the oceanic puffer *L. lagocephalus* recorded in San Sebastián (Lozano-Rey, 1952). Taking account of the location highlighted by Tortonese (1986), this could be a misidentification, but we cannot reject the possibility that he incorrectly placed the location on the map. Moreover, *E. guttifer* has never otherwise been reported in the French area of the Bay of Biscay (Quèro *et al*., 2008) or elsewhere in French waters (Béarez *et al*., 2017). No evidence of the presence of this species in the Bay of Biscay could be found, which raises doubts about the validity of this early record. The present record establishes a new fish species in Galician waters and constitutes the northernmost confirmed record in the eastern Atlantic Ocean.

The increase in number, as well as changes in the distribution of tetraodontiform fishes in the European Atlantic Ocean has been related to global warming (Bañón & Santás, 2011; Quéro *et al*., 2008). In Galician waters this is also supported by a rise of 0.24° C per decade observed since 1974 (Gómez-Gesteira *et al*., 2011) and by numerous occurrences of southern fishes, such as the red cornetfish *Fistularia petimba* Lacépède 1803 (Bañón &

Sande, 2008), the redbanded seabream *Pagrus auriga* Valenciennes 1843 and the bastard grunt *Pomadasys incisus* (Bowdich 1825) (Bañón *et al*., 2014), as examples of tropicalization of the north-eastern Atlantic ichthyofauna. A review of published data on north-eastern Atlantic fish species representing different biogeographic affinities, habitats and body sizes also supports the hypothesis that global warming results in a shift in the abundance and distribution of fish species (Rijnsdorp *et al*., 2009).

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TABLE 1 Measurement and meristic collected from a specimen of *Ephippion guttifer* from near Galicia, Spain

	Measurement (mm)	% L_S	Shipp (1974)
BIOMETRIC			
Total length (L_T)	570		–
Fork length (L_F)	529		–
Standard length (L_S)	445		106–402
Head length (L_H)	123	27.6	29.9–37.8
Preorbital length	43	9.7	14.5–18.2
Eye diameter	22	4.9	5.1–9.0
Postorbital length	58	13.0	–
Interorbital distance	61	13.7	12.3–16.6
Predorsal length	285	64.0	–
Dorsal fin base length	35	7.9	–
Preanal length	297	66.7	–
Anal-fin base length	28	6.3	–
Pectoral length	68	15.3	14.4–19.1
Body depth	140	31.5	–
Body width	97	21.8	–
MERISTIC			
No. of dorsal fin rays	10		9–11
No. of anal fin rays	8		8–10
No. of pectoral fin	19		18–20
No. of caudal fin rays	11		11
Mass			
Total mass (g)	2907		
Eviscerated mass (g)	2504		
Gonad mass (g)	32.5		

FIGURE 1 *Ephippion guttifer* caught in the Ría de Vigo, southern Galicia, north-west Spain (570 mm total length).

FIGURE 2 Distribution map showing georeferenced location points of *Ephippion guttifer* (●), the present record (●), and a probably invalid record (●). The inside map shows the exact location of the recorded point of the Galician specimen.

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2 Insert a double-headed arrow linking the green spot on the main map with the inset map.

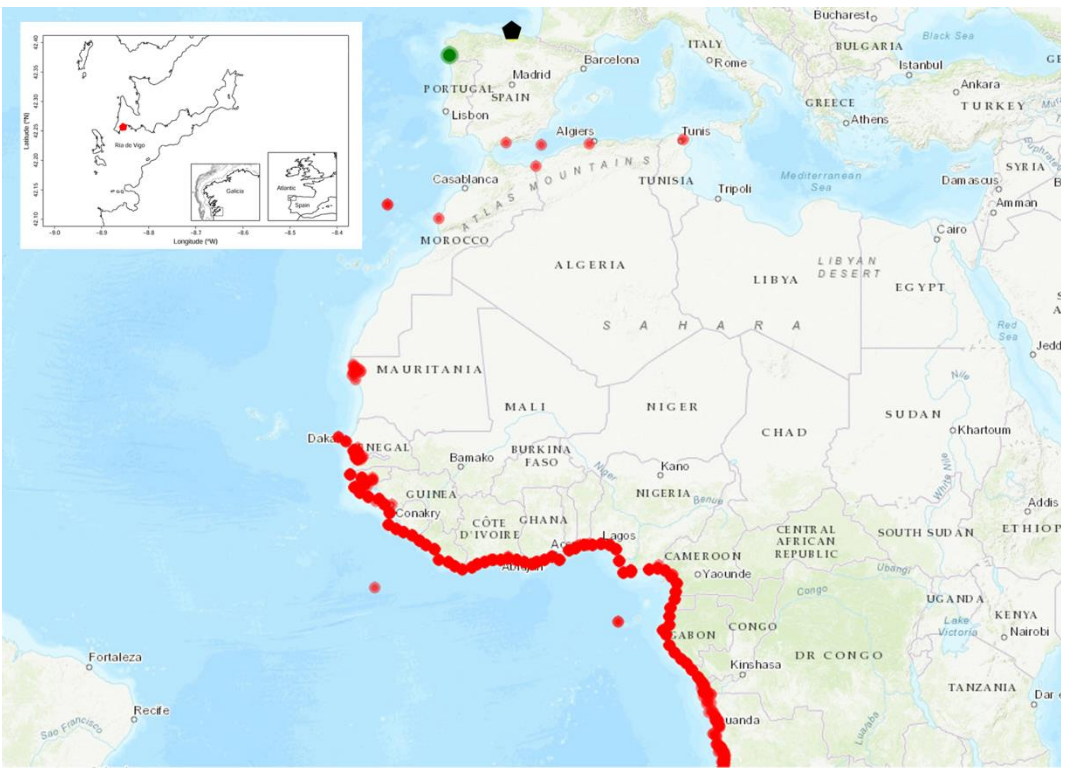

FIGURE 3 Morphological and histological views of the testes of *Ephippion guttifer*. (a) External morphology of the testes (scale in cm); (b) histological slide of the actively spawning testes with spermatozoa (Spz) filling the lumen of the lobules; (c) histological detail of the melanomacrophage centre (MC), spermatogonia (Spg) and spermatocytes (Spc); (d) histological detail of Spg, early spermatids (Spt1), advanced spermatids (Spt2) and Spz.

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1 Change a) to (a) etc.