Limnetica, 45(2): 000-000 (2026). DOI: 10.23818/limn.45.16

From the laboratory to the wild: Comprehending the reproductive dynamics of the Peloponnese Killifish *Valencia robertae* for its effective conservation

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Received: 13/01/25 Accepted: 16/06/25 Available online: 09/09/25

ABSTRACT

From the laboratory to the wild: Comprehending the reproductive dynamics of the Peloponnese Killifish *Valencia robertae* for its effective conservation

In depth knowledge of the reproductive ecology and ethology of a threatened fish species is needed for the design and implementation of successful conservation interventions, such as *ex situ* breeding and *in situ* translocation. In this *ex situ* study, we describe the critical parameters for successful reproduction of the threatened, endemic Peloponnese killifish *Valencia robertae* (Greece) and provide a full reproductive ethogram of the species that can guide successful captive propagation, appropriate habitat restoration and informed selection of suitable release habitats, and optimal release period. The Peloponnese killifish reproductive period is prolonged, lasting from early spring to early autumn, with spawning occurring mostly at night and mostly on gravel, with one egg per spawning event. Males exhibit breeding colouration and there are multiple behavioural interactions of males with females, as well as between males. In the Peloponnese killifish there is aggression in male-male interactions and no evidence of territoriality or parental care, through nest building or egg-guarding. These indicate male-male competition (mate monopolisation) with high levels of kleptogamy, mainly through sneaking ("parasitic spawning"), i.e., when other males try to fertilise the egg at the same time and, secondarily, through cannibalism, i.e., post-mating egg consumption by other males of different sizes. Although females are not selective about the size of males, there is a selectivity determined by the interaction of males, with the larger males driving off the smaller ones, which often act as satellites trying to mate secretly from the spawning male. We compare the reproductive behaviour of the killifish with other related genera with similar characteristics such as sexual dimorphism, male-female approach, and female substrate choice for egg deposition. Finally, we discuss future challenges to the killifish's reproduction within the frame of climate change and alien species' invasion, as well as the use of the ethogram in future conservation initiatives.

KEY WORDS: reproductive ethology, kleptogamy, breeding behaviour

RESUMEN

Del laboratorio a la naturaleza: Comprensión de la dinámica reproductiva del ciprinodóntido del Peloponeso* Valencia robertae *para su conservación eficaz.

Un conocimiento profundo de la ecología reproductiva y la etología de una especie de pez amenazada es necesario para el diseño e implementación de intervenciones de conservación exitosas, como la cría ex situ *y la translocación* in situ*. En este estudio* ex situ *se describen los parámetros clave para el éxito reproductivo del ciprinodóntido del Peloponeso (*Valencia robertae*), una especie endémica y amenazada de Grecia, elaborando un etograma reproductivo detallado que pueda guiar la propagación en cautiverio exitosa, la restauración apropiada del hábitat y la selección informada de hábitats de liberación adecuados y el período de liberación óptimo. El período reproductivo de* V. robertae *se extiende desde principios de primavera hasta principios de otoño. El desove ocurre mayoritariamente de noche, mayormente sobre grava, con una ovoposición unitaria por evento. Los machos presentan coloración nupcial distintiva y se identifican diversas interacciones conductuales, tanto entre machos y hembras como entre machos. Las interacciones macho-macho son marcadamente agresivas, aunque no se observa territorialidad ni cuidado parental, como construcción de nidos o protección de huevos. Este patrón sugiere competencia intraespecífica entre machos, predominando la cleptogamia. Este comportamiento incluye desove parasitario, donde otros machos intentan fertilizar el huevo simultáneamente, y canibalismo, es decir, el consumo de huevos tras el apareamiento. Aunque las hembras no muestran preferencia marcada por el tamaño del macho, las interacciones entre ellos favorecen a los de mayor tamaño, que desplazan a los más pequeños. Estos últimos adoptan roles satélites para intentar aparearse furtivamente con las hembras. El comportamiento reproductivo de* V. robertae *se compara con el de géneros afines, que también presentan dimorfismo sexual, cortejo e influencia de las hembras en la selección de sustratos para la ovoposición. Finalmente, se analizan los desafíos futuros para la reproducción y conservación de esta especie en un contexto de cambio climático e invasión de especies exóticas, destacando la necesidad de estrategias específicas, así como el uso del etograma en futuras iniciativas de conservación.*

PALABRAS CLAVE: *etología reproductiva, cleptogamia, comportamiento reproductivo.*

INTRODUCTION

Understanding the mechanisms of reproduction and breeding (i.e., mating behaviour, spawning mode, spawning substrate choice and larval development) is considered a necessary tool for the informed conservation of threatened species, such as creating insurance populations in the laboratory and reintroducing and/or enhancing wild populations, if deemed necessary (George et al., 2009; Kalogianni et al., 2022). The establishment of insurance populations can prevent the extinction of the species if, through some catastrophic events, all wild populations are lost (George et al., 2009). Furthermore, *ex situ* breeding in aquaria is increasingly recognised as important for the conservation of many freshwater fishes through informed *in situ* conservation interventions, ensuring their eventual success (Koldewey et al., 2013).

The threatened Peloponnese killifish *Valencia robertae*, endemic to southwestern Greece, is relatively recently described (Freyhof, Kärst & Geiger, 2014), and prior to its description, its populations were allocated to the Corfu killifish *Valencia letourneuxi*, encompassing southwestern and north western Greece killifish populations. Prior to their splitting, *Valencia letourneuxi* (Sauvage, 1880) was listed in Annex II of the Bern Convention as endangered and strictly protected species, and in Annex II of the European Union Habitats Directive 92/43/EEC as a priority species for conservation. *Valencia letourneuxi* is also listed as Critically Endangered by IUCN (Crivelli, 2006), while *V. robertae*, was recently evaluated by IUCN as Critically Endangered (Ford & Kalogianni, 2024). Both *V. robertae* and *V. letourneuxi*, are characterised by low density, fragmented populations in Greece, with their geographical range severely reduced in relation to the past (Kalogianni et al., 2010; Barbieri et al., 2015). More specifically, the southernmost populations of *V. robertae* are now regarded as extinct, while many westernmost populations of *V. letourneuxi* are also near extinction (Barbieri et al., 2015; Kalogianni et al., 2022). The decline of the two species has been mostly attributed to habitat degradation, and to the impacts of the alien Eastern mosquitofish *Gambusia holbrooki* (Girard, 1859) (Kottelat & Freyhof, 2007; Barbieri et al., 2015; Kalogianni et al., 2019). In a more recent study, the population trends of *V. robertae* and its sister species, *V. letourneuxi*, were assessed employing the largest compiled database to date (16 populations over the period 2005-2018), by applying the methodology of the Living Planet Index (LPI) that assesses average rate of change over time (Kalogianni et al., 2022). The LPI application revealed a dramatic decline of both species, with *V. robertae* declining by 91.0%. and *V. letourneuxi* declining by 97.7%. Water pollution, eutrophication and the alien Eastern mosquitofish *Gambusia holbrooki* (Girard, 1859) were the three best fitting predictors of their decline (Kalogianni et al., 2022). In this study, several conservation actions were proposed, including protection of the species' lowland spring habitats, habitat improvement through changes in water management and agricultural practices, mosquitofish invasion prevention and/or impact mitigation measures and *in-situ* and *ex-situ* conservation initiatives (translocations and *ex-situ* breeding). In the same work, several knowledge gaps were highlighted, notable among them the lack of information regarding the reproduction of these species, which could play a critical role in guiding future conservation strategies.

Reproductive behaviour in fish is dominated by male-male interactions, although in some cases females can also compete (Cavraro et al., 2013; Henson, et al., 1997; Altavilla et al., 2024). These complex male behaviours can be grouped into four categories of potential behavioural tactics: being quicker than rivals (scramble competition), monopolising resources, i.e., spawning sites, nests, or mates (resource or mate monopolisation), exploiting the monopolisation of resources or mates by others (reproductive parasitism), and/or finally co-operating or "trading" with resource holders (for a review see Tobolski, 2001 and references therein).

The first aim of this study was to identify reproduction temperatures, reproduction photoperiod, optimal physicochemical water parameters, type of spawning substrate, and reproductive period of the year, following a reproduction protocol of the threatened Peloponne killifish that can be applied for the creation of large "safety net" populations of this species, within a controlled aquaria environment. This protocol would involve target fish reproduction, with minimal mortalities and maximum hatching rates. The second aim of this work was to create a full reproductive ethogram of the species, enabling comparison with related species, but, most importantly, to foresee future challenges that may hinder the breeding of the species in the wild, as well as to inform future *in situ* conservation.

MATERIALS AND METHODS

Fish Breeding Facilities

For the purposes of this study, in September 2018, six aquaria, i.e., three aquaria of 350 L (dimensions 100x70x50 cm) and three aquaria of 280 L (dimensions 80x70x50 cm) were set at the HCMR laboratory premises. They consisted of 10 mm thick glass surfaces welded with aquarium silicone, suitable for living organisms. The aquaria were mounted on metal bases, painted with a non-toxic paint to prevent contamination by toxic metals. A 4-cm layer of washed dark-coloured river gravel (7–10 mm), was placed as substrate. Live plants and river cobbles were also added to reduce stress and provide shelter to the fish. Artificial lighting in the aquaria was provided by two T5, 39 W lamps that emit light suitable for aquaria, with daylight spectrum (8500 K), equipped with a timer as explained in the Fish Rearing section. The aquarium life support equipment consisted of a JBL Cristall profi e1501 filter with 16/22 mm hoses and pipes, intake strainer, elbow, bio-filter balls and bio-filter foam; water turnover was 1400 L/h, 20 W power, volume packaging 45.5 L. Air was supplied through a Sera air 275 R plus pump. Extra cooling units (Chiller- Conditioning unit Teco TK500) were added to the three 350 L aquaria, adjusted to 20 °C, and connected, after filtering, to a UV Vecton 600 TMC sterilisation system 25W UV Lamp.

To maintain a constant temperature in the smaller aquaria and to avoid overheating during the summer months from the operation of chillers, lamps, etc., a 12 000 BTU cooling unit was installed in the aquaria room, with an automatic re-powering system in case of power failure. Oxygen pumps and filters were connected to an uninterruptible power supply, so that they would not be affected by short power cuts.

Fish Collection

The killifish were collected from a spring-fed stream with a high-density Peloponnese killifish population (Mornos River Basin, Greece) in autumn to allow them to adapt to their new environment before the breeding season the following spring. Using a scoop net, a total of 709 fish were caught, of which 103 individuals belonged to the Peloponnese killifish *V. robertae*, 176 to the Western Greece goby *Economidichthys pygmaeus* and 429 to the Stymphalia minnow *Pelasgus stymphalicus* (26 trials, fished area 20.2 m^2). The physicochemical parameters of the stream water during fish sampling were as follows: conductivity 585 µs/cm, D.O. 9.27 mg/L, pH 7.5, salinity 0.27‰, temperature 17.6 °C. A total of 103 *V. robertae* individuals of various sizes were placed in small plastic bottles with water, pure oxygen from an oxygen tank and an ammonia-neutralising agent (Ammo Lock, API). The sympatrics were released into the water.

The fish were transported to lab aquaria the next day, with zero mortality during transport and acclimatisation. Ten days before the transfer, Nitrosomonas and Nitrobacter bacterial cultures (Sera Bio-Nitrivec) were added to the aquariums to enable the nitrification process through bacteria development. The physicochemical parameters of the aquarium water resembled those of the water in their natural environment, i.e., temperature 18.5 °C, pH 8.1 conductivity 653 µS/cm.

Fish Rearing

After acclimatisation, 16 adult female Peloponnese killifish and 22 adult males with Total Length (TL) of 18-35 mm were placed in a 350 L aquarium for the recording of reproductive behaviour (number kept low to minimise fish stress due to fish density and size range selected to study young adult fish of similar size per sex) and the rest of the fish (65 fish, TL 12 -25 mm) in a second 350 L aquarium. Water temperature was set at 20 °C (±1 °C), similar to the maximum summer water temperature in their natural habitat (Kalogianni et al., 2010a).

The aquarium lights were not switched on for the first 30 days in order to allow the fish to settle into their environment. There was only natural lighting in the room through a large (3 x 1 m) window facing westwards (natural light passed indirectly into the room, never reaching the aquarium directly). After the first month and the acclimatisation of the fish to their new environment with only natural lighting, artificial lighting was gradually introduced within a week, by, initially, switching it on two hours after sunrise and turning it off one hour before sunset and then gradually decreasing these two- and one-hour periods to reach the natural photoperiod values.

Chemical analysis of a water sample from the aquaria was performed once per month to measure nutrients and water ions (Table S1, Supplementary information, available at https://www.limnetica.net/en/limnetica). The parameters that were measured were: pH, Si mg/L, Ca mg/L, Mg mg/L, Na mg/L, K mg/L, HCO_3 mg/L, CO_3 mg/L, SO_4 mg/L, Cl mg/L, N-NO_3 mg/L, N-NO_2 mg/L, N-NH_4 mg/L, P-PO_4 mg/L, Total Hardness (mmol/L $CaCO_3$). Once per week a more cursory test was conducted, with pH and conductivity measured with a HANNA HI98129 pH/EC/TDS/ instrument, and ammonia and nitrates with JBL Testlab.

Provided the parameters were within the normal range (ammonia below detection level), a water change of 20% was performed every 30 days during routine removal of debris from the gravel substrate using suction. If water quality was found degraded, water changes were conducted more frequently. Water samples for chemical analysis were obtained and physicochemical measurements were made always before water change to detect degradation. Fish were fed once daily with frozen *Daphnia*, krill, copepods and Sera Vipan Nature flakes, and TetraMin Tropical Flakes *ad libitum*.

Reproductive study

At the end of February 2019, about a month before the start of the breeding season, natural plants were gradually replaced with artificial green synthetic wool "spawning mops" resembling plants that were easier to inspect for eggs. Three spawning mops were placed in the front part of the aquarium; two tied to a stone at the bottom and one tied to a plastic cork, floating at the surface, to assess fish preference for laying their eggs on surface plants or on the substrate. The spawning mops were checked daily for eggs (for more details on egg handling and rearing, see Kapakos et al., 2024a), which were collected and placed in a separate hatching tank.

A Wifi camera (Model Foscam C2), which features a night-time infrared camera was placed at a distance of one meter from the aquarium. Fish reproductive behaviour was recorded continuously on a 24-hour basis throughout the 2019 breeding season (recording period: 9/4/2019 –25/10/2019) and data were stored on a pc hard disk for further analysis). The breeding colouration of the fish was also documented photographically throughout the breeding season.

Video Analysis

Video material obtained from the aquarium with a total of 38 adult fish from a period of 120 h (31/5/2019 12:00 to 4/6/2019 12:00) was analysed initially in order (a) to identify spawning events (i.e., a sequence of behaviours culminating in a male and female assuming an S-shape posture at an angle to the substrate), (b) to distinguish whether each event took place during day or night-time (Fig. S1, Supplementary information, available at https://www.limnetica.net/en/limnetica) and (c) to determine the preferred type of spawning substrate (Total number of events 124). "Day" was set from 6:00 am to 9:00 pm since, the duration of the day in the summer in Greece exceeds 14.5 hours. *V. robertae* preference for spawning substrate [i.e., gravel, rocks and spawning mops ("artificial plants") either at the bottom or at the surface] was also assessed.

For the 124 spawning events detected during the 120 h surveyed, we recorded daytime/nighttime and substrate type. Of these events, 112 were further analysed for a series of behaviours (12 events took place at the back of the aquarium and thus behavioural interactions between fish were not clearly visible). These behaviours were selected after preliminary examination of the video material, following Malavasi et al. (2010) and Cavraro et al. (2013) with modifications (Table 1). These entailed male-male interactions and male-female interactions (no female-female interactions were observed), within a timeframe of 10 s before and after the spawning event.

Statistical Analysis

A two-sample Z-test of proportions was performed to examine differences in spawning activity between daytime and nighttime. This analysis was conducted using the prop.test function in R, which allows for hypothesis testing of equality of proportions among different groups. The function calculates confidence intervals and p-values for the comparison, providing a rigorous statistical framework for assessing whether the observed differences in spawning activity are statistically significant.

A Chi-square (X^2) test of independence was performed to examine differences in spawning activity across different substrate types. This analysis was conducted using the chisq.test function from the stats package in R, a widely used

Table 1. Behaviours involved in the Peloponnese killifish *V. robertae* reproductive sequence, quantified from the video material. *Comportamientos involucrados en la secuencia reproductiva del pez killifish de Peloponeso* V. robertae, *cuantificados a partir del material videográfico.*

Male-female	
Following	Spawning male follows the female (the male follows the female at a distance equal or less of one body length/ both moving)
Demonstrating	Spawning male assumes a semi- vertical position, with its head towards the substrate, demonstrating its dorsal area to the female
Male-male	
Fin/gill flaring	Spawning male flairs its fins/gills to other male (s)
Nipping	Spawning male nips another male (abrupt physical contact between the mouth of the male and the body of another male)
Sneaking	One or more "satellite" males attempt to also spawn with the female, while the breeding pair has assumed the S posture
Cannibalism	Egg cannibalism by another male

function for evaluating the association between categorical variables. The chisq.test function compares observed and expected frequencies in a contingency table, providing both the Chi-square statistic and the p-value to assess the significance of the relationship. This approach enables robust statistical inference about substrate preferences in spawning behaviour.

RESULTS

Breeding Colouration

At the beginning of March 2019, male fish started to exhibit breeding colouration (Fig. 1). During breeding, the ventral and lateral area of the males (below the lateral line) acquired an intense yellow colouration which covered part of the lower jaw, a large part of the operculum, and most of the abdomen. This colouration was also observed on the entire pelvic fins, most of the anal fin and occasionally on the posterior-ventral section of the caudal fin (Fig. 1a). Laterally, there were blue/grey stripes/bands, of various sizes, perpendicular to the body, which faded away to the yellow colouring at the ventral area. Light blue bands alternated with darker grey bands. There was cyan-blue colouration of the dorsal and caudal fin with a dark outline. Also, on the operculum, a black dot appeared only during the breeding season, which could remain visible from a few hours to a few days. Finally, the dorsal area of the males was darkly coloured (Fig. 1a).

By comparison, males before the breeding season (December 2018), had only a slightly yellow colouration on the anal fin, vertical body stripes, metallic-like colouration of the operculum, and lightly coloured dorsal area (Fig. 1b).

The female killifish did not exhibit any breeding colouration, with a greyish body with no stripes (Fig. 2). There were only chromatophores on the operculum with a metallic-like colouration, similar to that of the males.

Egg production

During the study period (2019), the first *V. robertae* egg was collected on 16/4/2019 and the last on 9/9/2019 (total number of eggs 17, always single eggs found on the mops). The onset and end of the reproductive period was also followed (with an interval on the covid 2020 year) in 2021 and 2022, i.e., in 2021 (total number of eggs = 25), the first egg of *V. robertae* was found on 11/5/21 while the last egg found and hatched was on 7/9/2021. In 2022 (total number of eggs = 29), the first *V. robertae* egg was found on 4/4/2022 and the last egg on 15/9/2022.

Reproductive time and substrate

Among the 124 recorded spawning events during the 2019 period, 47 occurred during the daytime and 77 during the nighttime. The Z-test analysis revealed a statistically significant difference in the proportions of spawning events occurring during the day versus night (x^2 = 13.565, ff=1, p =0.0036). Based on these results, the estimated probability of spawning during the daytime was 37.9%, whereas it increased to 62% during the nighttime.

In terms of substrate preference, 104 spawning events were observed on gravel, four on rocks, and 16 on spawning mops (restricted to those positioned at the bottom of the aquarium). The Chi-square analysis revealed statistically significant differences in the utilisation of the three substrate types for egg deposition (x^2 =137.1, df = 2, p=0.024).

Reproductive behaviour

A typical reproductive sequence in *V. robertae* entails initially a series of aggressive interactions between males, mostly gill flaring by opening of the operculum and fin flaring (Fig. 3a). During this interaction that lasts 2 to 4 s, the two males swim almost parallel to each other, and if one of the two does not move away, then there may be a nip.

Then the spawning male approaches the female; upon seeing the female near the substrate, the male usually approaches it from the side and rarely from the back (Fig. 3b). The male turns its head towards the substrate (in a position that looks like it is looking for food at the bottom) demonstrating to the female its dorsal area and trying to touch the female with its whole body.

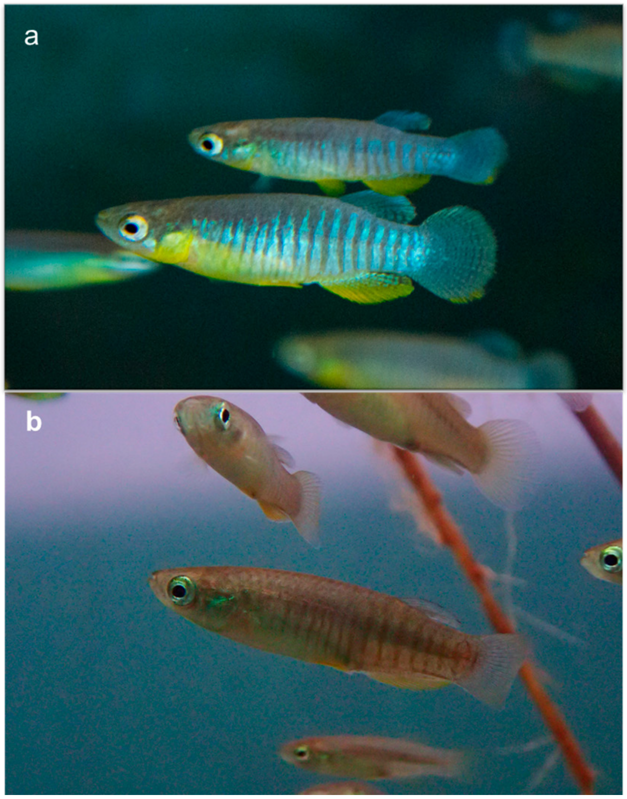

Figure 1. Male Peloponnese killifish with breeding colouration (July 2019) during (a) and prior to the repodiuctive period (b). *Macho de* V. robertae *en fase de coloración nupcial (Julio de 2019) observado durante el período de puesta (a) y en la fase previa al desove (b).*

Figure 2. Female Peloponnese killifish before the breeding season (December 2018). *Hembra de* V. robertae *antes del período reproductivo (Diciembre de 2018).*

Then the two fish swim together near the bottom.

Subsequently, the female positions itself at an angle to the substrate forming an S shape to which the spawning male synchronises, for 2 to 4 seconds; this process may stop abruptly if another male approaches, then a male-male aggressive interaction ensues. Also, the process can be interrupted at any moment by the female moving away. During spawning, another male or several males who are in proximity may approach to fertilise the deposited egg (sneaking, Fig. 4). Apart from sneaking, another variant of kleptogamy occurred that could be dubbed "fertilisation in momentous absentia" that involves fertilisation by a smaller male, while the larger male courting the female is momentarily engaged in chasing other males. Occasionally, another male approaches the breeding pair during spawning and attempts to cannibalise on the deposited egg (Fig. 5).

Concerning female-male interactions there was following and demonstrating in all events (100%) examined. Concerning male-male interactions, fin/gill flaring occurred prior to all

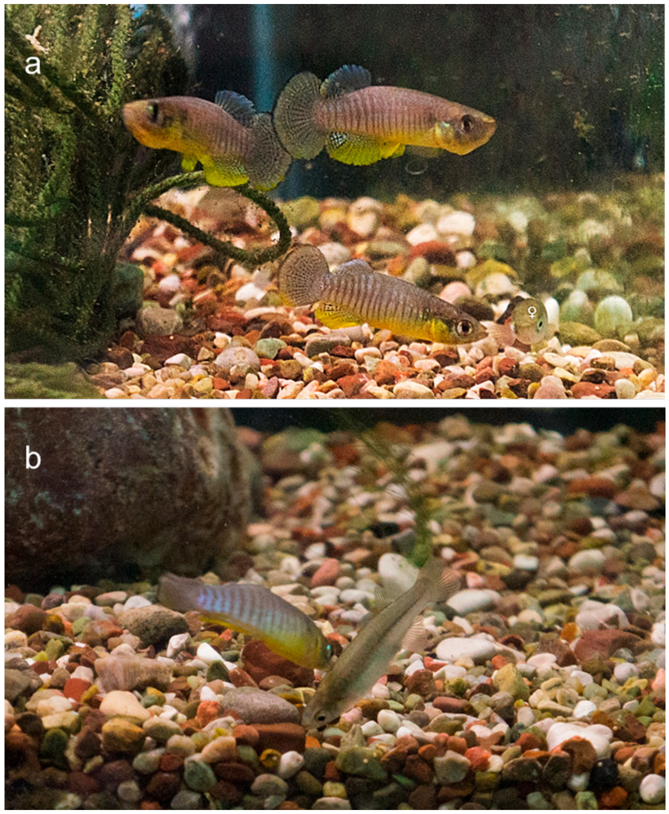

Figure 3. (a) Male-male Peloponnese killifish interaction during the 2019 breeding period. Three males compete for the female on the right, flaring their fins aggressively and opening their operculum. The black spot on the operculum is also visible. (b) A Peloponnese killifish male (left) approaches the female (right) prior to spawning on the gravel. *(a) Interacción entre machos de* V. robertae *durante el período reproductivo de 2019. Tres machos compiten por la hembra situada a la derecha, desplegando agresivamente sus aletas y abriendo los opérculos. También es visible la mancha negra en el opérculo. (b) Un macho de* V. robertae *(izquierda) se aproxima a la hembra (derecha) antes del desove sobre el sustrato de grava.*

Figure 4. A large Peloponnese killifish male, having chased away other large males, spawns with the female, with two smaller "satellite" males attempting also to spawn with the female (sneaking). *Un gran macho de* V. robertae*, tras haber ahuyentado a otros machos grandes, se aparea con la hembra, mientras que dos machos más pequeños, denominados "satélites", intentan también desovar con la hembra (desove por ocultación).*

spawning events examined (100%). Nipping was also recorded in all events examined, but in no case, was there visible injury from these interactions (clipped fins, loss of scales, etc.). Sneaking occurred in 74% of the events examined (in 83 out of 112 events), while cannibalism occurred in 15% of the events examined (in 17 out of 112 events), with a further 12 events where it was unclear whether there was cannibalism or attempt to spawn (Fig. 6). Fertilisation in momentous absentia of the dominant male was not quantified.

Neither territoriality nor parental care was observed. All males and females during the breeding season were swimming along the entire water column, with no indication of defending a specific area or of nest creation by the males or the females.

The reproductive ethogram of the Peloponnese

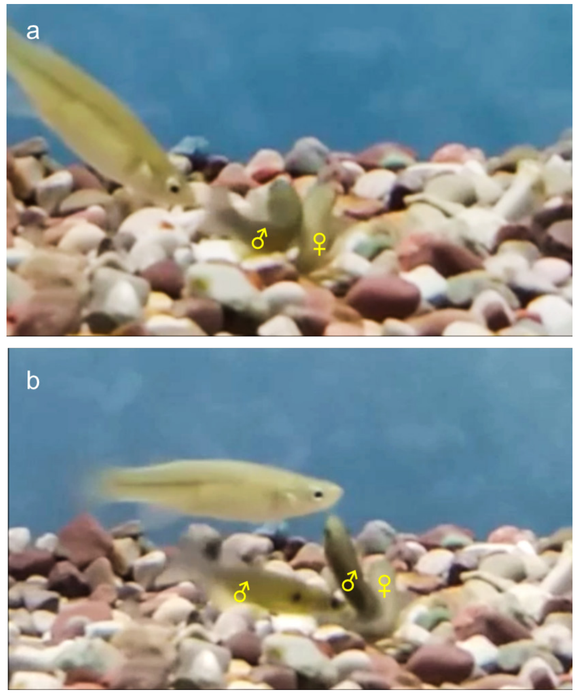

Figure 5. (a, b) As a Peloponnese killifish breeding pair spawns, a second male approaches to cannibalize on the egg deposited (consecutive video frames). *(a, b) Mientras una pareja reproductora de* V. robertae *desova, un segundo macho se acerca para canibalizar el huevo depositado (fotogramas consecutivos del video).*

Figure 6. During spawning, another Peloponnese killifish male often approaches the breeding pair, possibly to spawn. *Durante el desove, otro macho de* V. robertae *suele acercarse a la pareja reproductora, posiblemente para intentar desovar.*

killifish summarises the behavioural states within the species spawning sequence (Table 2).

DISCUSSION

The Peloponnese killifish was found to be a serial spawner, with females laying a single egg, with a preference for spawning at night and mostly on gravel. Diel patterns in spawning are identified as ecological adaptations with night or dawn/dusk spawning preference in freshwater fishes attributed to reduced predator pressure for the eggs by visual predators (Šmejkal et al., 2018). In addition, at early morning or late evening conditions at temperate streams and rivers for fish embryos and larvae are optimal, i.e., cooler temperatures and higher oxygen levels (Kondolf, 2000; Caissie, 2006). Similarly, spawning on gravel is possibly related to egg oxygenation, sediment filtration and predator protection as the eggs attached to gravel are oxygenated by the flowing water that also removes fine silt or sand and are better protected by visual predators (Mills, 1981; Kottelat & Freyhof, 2007). The Peloponnese killifish also exhibited intense sexual dimorphism during the breeding season and their reproductive behaviour is characterised by intense male-male competition that indicates strong mate monopolisation and some reproductive parasitism. More specifically,

Table 2. Reproductive Ethogram of the Peloponnese killifish. *Etograma reproductivo del Cyprinodonto del Peloponeso*

Behavioural patterns	Description	Duration	Comments
Pre-Reproductive Recognition (Courtship/ Display)	The females are moving close to the bottom of the aquarium, examining the substrate. The males acquire breeding colouration throughout the breeding season (spring-summer).		If a male approaches the female, it usually quickly moves away.
Pre-reproductive Dominance	The large male follows the female trying to be constantly close to the female. When another male approaches, both males open their operculum, slightly arch their bodies and approach each other in parallel and at a close distance until one of the two moves away. In a few cases, a male has been observed headbutting its opponent (females do not exhibit any aggressive behaviour towards each other).	2-10 seconds	No fin or body injuries were observed macroscopically.
Spawning: egg laying	When the female finds a suitable spot, it tilts its body at a 45° angle to the substrate and assumes an "**S**" posture with its head upwards.	2-3 seconds	During substrate inspection by the female, in some cases the large male swims along with the female lightly touching its body.
Spawning: fertilisation	The female's **"S"** posture is the reproductive signal for the male to also assume this posture and fertilise the egg.	2-3 seconds	Once the female assumes the **"S"** posture, several other males' approach and attempt to fertilise the egg.
Reproductive klepto-gamy	During fertilisation, other males of similar size as well as male satellites appear, attempting to spawn with the female. In some cases there is cannibalism of the egg by the other males.	2-3 seconds	
Post-reproductive: Parental Care	No care of the eggs after fertilisation by either the male or the female spawner.	Incubation period is 18 days at 20 ±1°C	Eggs have negative buoyancy and filaments at the outer surface which enable them to be attached to stones and plants.

fin flaring, the parallel positioning of the body of the males to project the breeding colouration in full extent in a lateral display and gill flaring by opening the operculum were all low- aggression behaviours used in male-male only interactions, and observed only during the mating season.

Aggressive mate monopolisation and reproductive parasitism

Male-male interactions were very similar to those observed in the related Mediterranean killifish *Aphanius fasciatus* (Cavraro et al., 2013 & Malavasi et al., (2010). More specifically, several similarities were found in the Peloponnese killifish reproduction with the reproduction of the killifish *Aphanius fasciatus*, recently described by Altavilla et al. (2024), such as the high levels of competition between males with an intense sexual selection regime of males competing to monopolize the female. The intense display of males to other males with the aim of chasing them away (i.e., "flashing") was also recorded in the Waccamaw killifish *Fundulus waccamensis* (Hubbs & Raney, 1946), which has also the same breeding colouration of blue lateral stripes and yellow abdominal area, as the Peloponnese killifish. However, *F. waccamensis* male-male interactions, as well as those of the African killifish *Aphyosemion gardneri* (Boulenger, 1911) are much more aggressive, as they include 'tail beating' (Shute et al., 1983), a severe mechanical stimulus which can forcefully displace the opponent (Kroll et al., 1981); this behaviour is probably related to the territoriality of the species, a behaviour never recorded in the Peloponnese killifish. Furthermore, in the current study, there was no apparent injury during male-male interactions; exhaustion and injuries have been reported in the literature in some annually living killifishes (killifishes that live in lakes that dry up in summer), which can be attributed to the urgency to reproduce before they die (Passos et al., 2014). There were also similarities in male-female approach with the Waccamaw killifish, as well as other Fundulus species, such as “dipping” in males (male swimming slowly forward with its head pointed downwards), a behaviour similar to ‘demonstrating’ to the female in the current study. Also, just prior to spawning, the Waccamaw killifish female tilted its head downward and ‘nipped’ at the substrate (took a mouthful of sand and spat the grains in front of it), possibly to test the suitability of the substrate for egg laying; in the Peloponnese killifish, an exploratory behaviour of the female, moving very close to the substrate prior to spawning, was also observed.

Size selection has been reported in annual killifishes, with females choosing the largest males at the beginning of the breeding season; however, as the drying up of their temporary habitats approaches this preference decreases (Passos et al., 2014). It appears that Peloponnese killifish females are not selective about the size of males; however, based on the current observations, larger males prevailed in male-male interactions and could more easily chase away smaller males. Smaller males however were opportunistic and could mate with a female, while two larger males were engaged in aggressive display.

Cavraro (2013) studying the related Mediterranean killifish *A. fasciatus* identified two forms of kleptogamy, termed ‘sneaking fertilisation’, i.e., the simultaneous spawning of two or more males and ‘post-mating cannibalism’, i.e., egg-eating by peripheral males, both behaviours also observed in the Peloponnese killifish. Sneaking as observed in the Peloponnese killifish at high frequency, involves the dominant male initiating the S-shape process to fertilise the egg (when the male-male interaction stops for seconds) and other male(s) appearing trying to take advantage and fertilise the egg.

There are several examples of an ontogenetic transition from parasitism to mate monopolisation as the males grow (Taborsky, 2001), which it is assumed is also the case in the Greek killifishes. Cannibalism was also recorded in the Peloponnese killifish, at low frequency, in this study. A dietary study of the Peloponnese killifish has shown indications of cannibalism as killifish eggs were found in the fish gut (Kalogianni et al., 2010b), while high degrees of cannibalism have been reported also in the related species, *V. hispanica* (Rincón et al., 2002).

Finally, multiple paternity is very common in natural fish populations, while multiple motherhood occurs less frequently (Coleman & Jones, 2011). In our study, each female killifish released a small number of eggs during a spawning season (one egg per spawning event), usually, in the presence of several competing males. Competition among males and their attempt at egg fertilisation possibly increases genetic diversity in the offspring, providing evolutionary advantages (Coleman & Jones, 2011), but see Karl, 2008). An important prerequisite for increased genetic diversity from multiple paternity is that females are not monopolised by a few males and that the fish population is not small or isolated (Coleman & Jones, 2011). In addition, nest building and its supervision by a male, limiting its participation in future reproductive events, is correlated with low rates of multiple paternity (Coleman & Jones 2011). In the case of the Peloponnese killifish no nest building or further parental care was observed, and no monopolisation of egg fertilisation by a few dominant males. On the contrary, a broad reproductive strategy was observed, such as male satellites (sneaking), many males attempting to spawn with a female, etc. Another feature of the reproductive strategy through multiple paternity is that it requires a greater energy investment by males in sperm production (Fitzpatrick, 2020). The energy investment by the males in our case is evident in the intense nuptial colouration, in the constant male chasing and in male-male confrontations.

Spawning opportunism and breeding colouration

The Peloponnese killifish performed spawning on a variety of substrates, but mostly on gravel rather than on the mops. Also, there was no attempt by the females, that initiate the actual spawning by first assuming the S posture, to inspect the mops for a longer time than the gravel of the aquarium. Furthermore, *in situ* studies have shown that the Greek killifishes prefer karstic spring-fed habitats with floating and emergent vegetation, as well as clean fine gravel (Kalogianni et al., 2010a). Given that the eggs are equally adhesive to plants and harder surfaces such as gravel, it is evident that the species is opportunistic in terms of its spawning substrate.

Concerning the breeding colouration, it is not possible to fully decipher the exact role of the black spot in the operculum. This secondary male sexual characteristic, however, is possibly similar to the black stripe on the caudal fin of *A. fasciatus* (Altavilla et al. 2024), and as in the case of the black stripe in *A. fasciatus*, all males regardless of the presence of the black spot participated in courtship, spawning and aggressive interactions. Altavilla et al. (2024) also reported that the black stripe in *A. fasciatus* is associated with increased male aggressive behaviour; in our study, observations indicated aggressive interactions both between males with dots as well with males without dots. Generally, however, the males that had a more intense black spot had a more erratic/aggressive behaviour towards other males, though this was not quantified. Furthermore, in the few cases where aggressive interactions between males culminated to biting or headbutting the opponent's body, both fish or, more rarely, one of the two, had displayed the black spot. Also, the dots faded during the maintenance and cleaning of the aquariums, when the aggressive interactions between males subsided. It thus appears to be a complementary or independent colouration, but not necessary for the achievement of the reproductive process. In contrast, the dark colouration on the dorsal area of the males, may be of importance in the male-female interaction, as the male approaches the female with its head pointed downwards.

Greek killifishes start to breed in early spring and continue reproduction until the end of summer. They deposit their eggs, which are relatively large in diameter for the size of the fish (Kapakos et al., 2024a), on plants, gravel, and stones without providing any parental care. According to the classification of Teletchea et al. (2009), the Peloponnese killifish is closer to the group of early-spring spawners with no parental care, while according to the classification of Vila-Gispert & Moreno-Amich (2002) this species could be characterised as opportunistic, due to early maturity, low fecundity, small body size, and prolonged breeding season.

Future challenges and lessons for conservation

The target species reproduced successfully at 20 °C; in nature, the minimum and the maximum temperature recorded in Peloponnese killifish habitats during the breeding season were 16.1 °C and 22 °C respectively (Kalogianni et al., 2010a). Under current climate change scenarios, an increase in the water temperature of aquatic habitats that may seriously impact the reproduction of the Peloponnese as well as of the Corfu killifish is foreseen. Their sister species *Valencia hispanica* (Valenciennes, 1846), discontinues breeding in outdoor ponds when water temperature exceeds 27-29 °C in the summer (V. Gallego Albiach and P. Risueño, personal communication). Thus, a preference for spring-fed habitats as "thermal refugia" could partially explain the aggregation of the Peloponnese and the Corfu killifish in spring-fed habitats during their prolonged mating season. These karstic habitats that are also characterised by clean, well oxygenated waters with abundant aquatic vegetation are thus crucial for the survival of the species and the conservation of these habitats is a priority (Kalogianni et al., 2010a). There is ample evidence on the effects of climate change on fish reproduction and early life history stages (for a review of climate change effects on fish reproduction see Pankhurst & Munday, 2011 and, for a wider discussion, the more recent Nagelkerken et al., 2023 and Lema et al., 2024); however, our hypothesis that water temperature increase may negatively influence our target species' reproduction, should be tested under controlled laboratory conditions.

The multilevel, complex breeding ethology of the Greek killifishes may be negatively affected by the presence of the alien species *Gambusia holbrooki* (Girard, 1859), since in a recent study it has been demonstrated that the mosquitofish exhibits aggression towards the Greek killifishes (Kapakos et al., 2024b). In the Peloponnese killifish, the observed pattern of monopolisation among males was dominant as well as the less frequently observed behaviour of reproductive parasitism. Competition for mates and fertilisation often involve fighting behaviour and conflict

(Tobolski, 2001) that has a high energetic cost for the male fish. Aggression by the mosquitofish can cause additional energetic costs to the killifishes and a reduction of mate monopolisation, the loss of food resources, and the reduction of the ability to explore new environments to locate new females. In mesocosm experiments it has been shown that mosquitofish aggression can disrupt courtship in the related species *V. hispanica* (Rincon et al., 2002). This may lead to increased reproduction by smaller males through adaptations to reproductive parasitism. If this type of breeding prevails, genes conferring less aggressive competition, smaller overall size and less intense colouration in males may prevail. On the other hand, mosquitofish aggression may interfere with female killifish in their quest for suitable substrate to lay their eggs, through chasing or biting. In summary, mosquitofish aggression may have both direct negative impacts by disruption of mating as well as long term impacts through a change in male phenotypes (Tabrovsky, 2001).

Concerning the use of our and similar studies in freshwater fish conservation, it should be noted that a detailed reproductive ethogram is essential for successful *ex situ* breeding and *in situ* translocations, as it provides a detailed behavioural roadmap that ensures reproduction can occur naturally or be assisted effectively under both controlled *ex situ* or natural/restored *in situ* conditions (Brown et al., 2013). More specifically, it can help identify key behaviours and the appropriate space, substrate, social structure, and sex ratios in aquaria for successful propagation. It can also be critical in the design of breeding tanks, e.g., in the case of *V. robertae* and related species that exhibit egg cannibalism, equipped with egg traps or removable substrates (Miano et al., 2019). The ethogram can also indicate the optimal day period to monitor reproductive behaviour in aquaria and to carry out egg sourcing, which in the case of our target species, should be concentrated in night hours. Knowledge of spawning periodicity also implies that lighting and temperature regimes in *ex situ* facilities should, as in the case of *V. robertae*, replicate the natural cycle. It is also widely accepted that knowledge of preferred spawning substrate can determine habitat suitability in both artificial settings, i.e., propagation in aquaria with substrate that mimics optimal natural conditions for spawning and egg/larvae development (Malinovskyi et al., 2018), as well as in natural settings i.e., *in situ* fish translocations in release habitats with appropriate substrate as well as temperature and flow conditions (Taylor et al., 2019).

Finally, the ethogram can improve the success of a planned *in situ* translocation by guiding habitat restoration (e.g., interventions to provide appropriate spawning substrate) prior to the translocation, as well as by dictating the optimal time window for the release to ensure successful population establishment. In conclusion, without a full reproductive ethogram, conservation efforts risk failure due to mismatched environments, mistimed releases, and/or overlooked behavioural needs of the target species (Rakes et al., 1999; George et al., 2009; Sousa-Santos et al., 2014).

ACKNOWLEDGEMENTS

The authors wish to thank P. Kouraklis for assistance in fish collection and S. Laschou for chemical analyses. This work was conducted within the frame of project DECAGON funded by the A.G. Leventis Foundation and the Zoological Society of London (ZSL). This work forms part of the PhD thesis of Y. Kapakos at the Laboratory of Applied Hydrobiology, Department of Animal Science, Agricultural University of Athens (AUA), Greece. The Hellenic Centre for Marine Research (HCMR) had secured all necessary permits for fish collection from the Greek Ministry of Environment, Energy and Climate Change (permit 9ZE24653Π-ZO6, 20/7/2016; the HCMR Research Ethics Committee was still under development when this research was conducted). Fish handling in the field and the laboratory at HCMR complied with Greek guidelines on the protection of animals used for scientific purposes (Official Journal of the Greek Government No. 106/30 April 2013), where applicable.

CREDIT AUTHOR STATEMENT

K.Y.: Conceptualization, Methodology, Data curation, Writing- Original draft preparation; V.K.: statistical data analysis, spanish Translation, text editing; Z.B.: contribution to text writing, text editing; K.N.: contribution to text writing, text editing; K.E.: Conceptualization, Contribution to methodology, text writing and editing, supervision.

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