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Feeding habits and reproductive biology of the introduced fish *Pseudoxiphophorus bimaculatus* (Cyprinodontiformes: Poeciliidae) in a spring in central Mexico

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ABSTRACT

Introduction: *Pseudoxiphophorus bimaculatus* is a fish native to the Mesoamerican Atlantic slope from Mexico to Nicaragua. This species has been introduced into aquatic ecosystems in central Mexico, impacting biodiversity through competition, predation and habitat modification.

Objectives: To evaluate the trophic strategy and reproductive biology of the introduced *P. bimaculatus* in San Sebastián Spring, West-Central, Mexico.

Methods: Fish were captured using minnow traps and seine nets throughout an annual cycle and categorized by size, season, and sex. Samples were collected bimonthly at one sampling site between April 2019 and February 2020.

Results: A total of 264 individuals of *P. bimaculatus* were studied. Their diet comprised 17 items, with remains of terrestrial insects being the most consumed (29 %). The species exhibits a relatively narrow trophic niche as a carnivorous-insectivorous secondary consumer (Trophic level (TROPH) = 2.89 ± 0.36). Significant differences in diet were observed between the sexes. The sex ratio varied seasonally from 1:1 to 2:1 (female:male), and reproduction occurs year-round. The average fecundity was 21 embryos per female. A correlation was identified between fecundity and standard length. Males reach maturity earlier at 34 mm Standard Length (SL), while females mature later at 54 mm SL.

Conclusions: *P. bimaculatus* exhibits a predominantly insectivorous diet and maintains continuous reproductive activity throughout the year. The combination of *P. bimaculatus* trophic and reproductive traits suggests a capacity to exploit locally available resources and persist under variable environmental conditions, associated with successful establishment in non-native environments. Further research is needed to evaluate potential interactions between *P. bimaculatus* and native fish species and their implications for freshwater biodiversity in central Mexico.

Key words: conservation; invasion ecology; lentic ecosystems; non-native poeciliids.

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RESUMEN

Hábitos alimentarios y biología reproductiva del pez introducido *Pseudoxiphophorus bimaculatus* (Cyprinodontiformes: Poeciliidae) en un manantial de México centro

Introducción: *Pseudoxiphophorus bimaculatus* es un pez nativo de la vertiente atlántica mesoamericana desde México hasta Nicaragua. Esta especie ha sido introducida en ecosistemas acuáticos en el centro de México, impactando la biodiversidad por competencia, depredación y modificación del hábitat.

Objetivos: Evaluar la estrategia trófica y biología reproductiva de *P. bimaculatus* introducido en el Manantial de San Sebastián, centro-oeste de México.

Métodos: Los peces fueron capturados utilizando nasas y redes de arrastre a lo largo de un ciclo anual y categorizados por talla, temporada y sexo. Las muestras fueron recolectadas bimestralmente en un sitio de muestreo entre abril 2019 y febrero 2020.

Resultados: Se estudiaron un total de 264 individuos de *P. bimaculatus*. Su dieta comprendió 17 artículos, siendo los restos de insectos terrestres los más consumidos (29 %). Esta especie exhibe un nicho trófico relativamente estrecho como consumidor secundario carnívoro-insectívoro (Nivel trófico (TROPH) = 2.89 ± 0.36). Se observaron diferencias significativas en la dieta entre los sexos. La proporción sexual varió estacionalmente de 1:1 a 2:1 (hembra:macho) y la reproducción tiene lugar durante todo el año. La fecundidad promedio fue de 21 embriones. Se identificó una correlación significativa entre la fecundidad y la longitud estándar. Los machos alcanzan la madurez antes a los 34 mm Longitud Estándar (LE), mientras que las hembras maduran más tarde a los 54 mm LE.

Conclusiones: *P. bimaculatus* presenta una dieta predominantemente insectívora y mantiene una actividad reproductiva continua durante todo el año. La combinación de características tróficas y reproductivas de *P. bimaculatus* sugiere su capacidad para explotar los recursos locales disponibles y persistir en condiciones ambientales variables, asociado con un establecimiento exitoso en ambientes no nativos. Se necesita más investigación para evaluar las posibles interacciones entre *P. bimaculatus* y las especies de peces nativas, así como sus implicaciones para la biodiversidad de los ecosistemas dulceacuícolas en el centro de México.

Palabras clave: conservación; ecología de la invasión; ecosistemas lénticos; poecílidos invasores.

INTRODUCTION

Central Mexico is a region for conserving fish diversity, hosting around 98 native species, of which 70 % are endemic (Domínguez-Domínguez et al., 2006, Domínguez-Domínguez et al., 2008; Miller, 2009). In contrast, over 25 exotic species have also been reported (Contreras-MacBeath et al., 2014; González-Martínez et al., 2014), some of which are invasive and impact aquatic ecosystems and biodiversity (Lowe et al., 2000).

Introducing and establishing non-native species pose a global threat to biodiversity, often resulting from intentional or accidental human releases (Lockwood et al., 2013; Barroso-Magalhães et al., 2023). In Central Mexico, this phenomenon is considered one of the leading causes of the decline of native fish species (Domínguez-Domínguez et al., 2006; Domínguez-Domínguez et al., 2008; Gesundheit & Macías, 2018). The impact of invasive species on native fish populations may result from their strong ability to compete for resources, their successful establishment of new populations, and the introduction of pathogens (Deacon et al., 2011; García-Vázquez et al., 2017; Pyke, 2008). These factors often interact with other human-induced issues, such as pollution and habitat destruction, further harming freshwater ecosystems (Dudgeon, 2006; Mendoza-Alfaro & Koleff-Osorio, 2014). In Central Mexico, non-native species are among the major threats contributing to the increased extinction risk of native species (Lyons et al., 2019; Webb & Miller, 1998). Freshwater ecosystems in Mexico have historically received limited attention and conservation investment, despite the adverse effects of non-native fish species, placing native species at a critical risk of extinction (Lyons et al., 2019; Zambrano et al., 2006).

One notable invasive species is *Pseudoxiphophorus bimaculatus* (Heckel, 1848), commonly named the Twospot livebearer. This species is classified within the family Poeciliidae and has a natural distribution along the Atlantic slope of Central America, ranging from the Misantla River in Mexico to the Prinzapolka River in Nicaragua (Miller, 2009). In its native habitat, it is recognized as an herbivorous species (Vega-Cendejas, 1997); however, outside of this range, it has been identified as a carnivorous-insectivorous species, primarily consuming resources of animal origin (Carbajal-Becerra et al., 2020; Mar-Silva et al., 2021; Trujillo-Jiménez & Toledo-Beto, 2007). Within its native range, *P. bimaculatus* exhibits a balanced sex ratio (1:1), characterized by slow growth and delayed maturation, with females being larger than males (Olinger et al., 2016). In contrast, in its non-native distribution range, the sexual ratio is favored by females, with rapid growth to maturity, and females being larger than males (Ramírez-García et al., 2018).

P. bimaculatus has been widely introduced throughout Central Mexico because of aquaculture activities, including accidental releases associated with stocking practices, escapes from aquaculture systems and forage fish (Carbajal-Becerra et al., 2020; Contreras-MacBeath & Ramírez-Espinoza, 1996, Contreras-MacBeath et al., 2014; Espinosa-Pérez et al., 1993). It has been identified as a successful competitor against native species (Magurran, 2009; Ramírez-Carrillo & Macías-García, 2014; Suárez-Rodríguez et al., 2023), threatening freshwater systems by altering biodiversity and ecosystem functioning. Trophic dynamic in freshwater fish assemblages is typically structured by species occupying relatively well-defined niches, where resource partitioning reduces interspecific competition and contributes to community stability (Cucherousset & Olden, 2011; Ross, 1986). Its ability to tolerate a wide range of environmental conditions, females are able to retain active sperm in ovarian tissues and to bear successive broods without reimpregnation, and its capacity to exploit various food resources may facilitate its establishment and persistence, thereby increasing competitive pressure on native species and contributing to shifts in trophic dynamics within invaded ecosystems (Carbajal-Becerra et al., 2020; Barroso-Magalhães & Jacobi, 2017; Mar-Silva et al., 2021).

In Western Central Mexico, *P. bimaculatus* has been introduced into the Teuchitlán River (Ramírez-García et al., 2018) and the San Sebastián Spring, Jalisco, Mexico both freshwater systems situated in the upper section of the Ameca River basin—an area of significant interest for the conservation of endemic freshwater fish (Domínguez-Domínguez et al., 2008). San Sebastián is a natural spring that was artificially dammed and is currently used mainly to supply water to livestock and agriculture (Ruíz et al., 2014). Research on the ichthyofauna in this area is limited, but seven native species have been documented (*e.g.* Piller et al., 2015; Domínguez-Domínguez et al., 2016). Among these, three species persist, including the San Marcos redtail splitfin *Xenotoca doadrioi,* an endemic fish that has largely disappeared from its original range, and therefore its conservation status is considered critically endangered (Koeck. 2019). Furthermore, four no native fish have been established on the site: tilapia *Oreochromis* sp., green swordtail *Xiphophorus hellerii*, Southern platyfish *Xiphophorus maculatus,* and *P. bimaculatus* (Domínguez-Domínguez et al., 2016). Consequently, the San Sebastián Spring is recognized as a vital water body for conserving freshwater fish within the upper Ameca River basin (Domínguez-Domínguez et al., 2016).

Therefore, the study of the diet and reproductive biology of *P. bimaculatus* in the San Sebastián Spring—a crucial water system for the conservation of at least one critically endangered fish species—is vital for understanding the invasive potential of this species, its trophic interactions, and its potential negative impact on native species. Examining its reproductive biology can provide insights into its life cycle, invasion stage, and reproductive potential. The objectives of this study are to 1) evaluate the feeding habits, and 2) characterize reproductive

traits, including sex ratio, fecundity, size at firs maturity, and reproductive seasonality of the introduced species *P. bimaculatus* at the San Sebastián Spring in Central Mexico.

MATERIALS AND METHODS

The San Sebastián Spring is situated in the town of San Sebastián (formerly the Ex-hacienda San Sebastián) within the municipality of Etzatlán, state of Jalisco. This freshwater ecosystem is a spring covering an area of approximately 6 375 m^2 (20°49'27.0" N, 104°07'9.1" W) (Fig. 1).

A preliminary analysis was conducted to determine the number of samples required to accurately represent the diet of *P. bimaculatus* following the methods proposed by Mar-Silva et al. (2021) (SMF 1). We established size classes using Sturges' rule (Sturges, 1926) to ensure a standardized grouping of individuals. These classes were subsequently evaluated in relation to diet and reproductive parameters based on preliminary data using ANOVA tests (Mar-Silva et al., 2021). The resulting size classes were consistent with biologically meaningful differences among individuals, particularly in terms of ontogenetic variation, including growth-related changes in diet and reproductive condition. Accordingly, three size classes were determined for each sex: For females, size I (15.71-42.93 mm), size II (42.94-57.62 mm) and size III (> 57.63 mm); for males, size I (17.29-28.50 mm), size II (28.51-39.71) and size III (> 39.72).

Collections of *P. bimaculatus* were carried out bimonthly during an annual cycle from April 2019 to February 2020. The fish were collected using a seine net (0.5 cm mesh, 10 m long and 1.2 m wide) and minnow traps (Gee-minnow-traps® G-40, EE. UU. Stainless steel, square mesh 0.5 m, cylindrical, 42 cm long, and 19 cm in diameter, with two 2.5 cm holes with inverted cone inlets). The traps

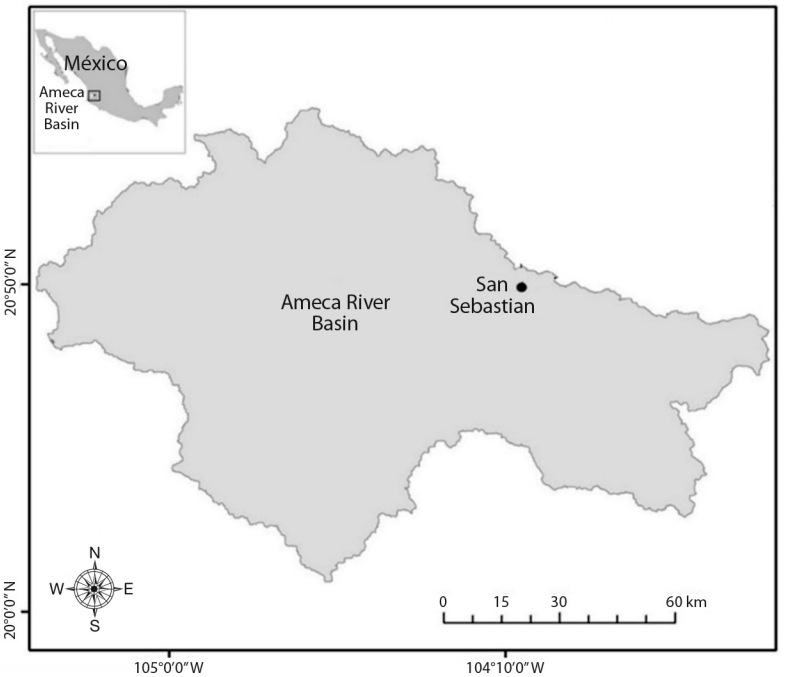


Fig. 1. Geographic location of the study area. The map shows the location of Ameca River basin in Central Mexico and the sampling site (San Sebastián Spring) in the municipality of Etzatlán, Jalisco.

were set for a one hour per sampling event to standardize sampling effort and minimize potential biases associated with prolonged trap exposure, such as fish mortality, following general recommendations for passive fish sampling methods (Herrerías-Diego et al., 2019; Hubert et al., 2012). Collected organisms were euthanized with benzocaine at 75 mg/L, based on concentrations applied to fishes of similar size (Rueda-Jasso et al., 2017), and in accordance with general guidelines for euthanasia of fishes using anesthetic agents (American Veterinary Medical Association, 2020). Specimens were subsequently preserved in 4 % formalin and transported to the laboratory. The Standard Length (SL) was measured in millimeters (mm) with a vernier (Truper® CALDI-6MP, precision 0.01 mm). The wet weight of the organisms was recorded (g) with a semi-analytical balance (VELAB VE-300, precision 0.01 g). Dissection of each individual was performed through an incision in the ventral region to extract the stomach and gonads, which were then weighed with the analytical balance. Gastric repletion was documented following the criteria of Borghetti et al. (1994). Semi-permanent preparations were subsequently made using the stomach contents of the individuals (10 x 10 square grid, dimension 1.8 mm x 1.8 mm). An optical microscope (Axiostar Plus, Carl Zeiss) was used to identify the components of the stomach content and quantify its contribution to the diet.

Trophic analysis: The feeding habits were analyzed through both qualitative and quantitative methods. For qualitative analysis, the different components found in the stomachs of the organisms were identified using the taxonomic keys of Merrit & Cummins (1996) and Merrit et al. (2019) for insects, as well as Thorp and Covich (2010) for other macroinvertebrate groups. Any ingested food that could not be identified was recorded as unidentified organic matter. A modification of the quadrant method (Hynes, 1950) was used for the quantitative analysis. We evaluated the percentage of area (PA) by quantifying the area of each food item in relation to the total stomach content. To reduce potential observer bias and allow objective and standardized estimation of prey, all area measurements were performed using a microscope-mounted digital camera (Amscope, MD-35) with a calibrated measurement scale using the software AmScope 3.7. We also assessed the frequency of occurrence (% FO) of each component of stomach content by calculating the number of stomachs in which a specific item was present relative to the total number of stomachs analyzed, expressed in percentage (Stark & Schroeder, 1971). The Index of Relative importance (IRI) was obtained as a measure of the importance of a particular component of the stomach content in the diet (Cortés, 1997), using the equation IRI = FO*PA/100, where FO is the frequency of occurrence, and PA is the percentage of area.

The IRI was expressed as IRI % to facilitate the visualization of the diet and allow the comparison of the stomach content articles, using the following correction (Cortés, 1997):

$$IRI\ \% = \frac{100 \times IRI_i}{\sum_{j=1}^{n} IRI_j}$$

The trophic level (TL) of the species was evaluated using the TrophLab database program (Pauly et al., 2000) through the equation:

$$\mathrm{TL_i} = 1 + TL_j = 1 + \sum_{j=1}^{nG} DC_j \times TROPH_j,$$

where DC_j represents the proportion of prey j in the diet of species i and $TROPH_j$ is the TL of prey j. Niche breadth was estimated using Levin's niche breadth index (Krebs, 1989) to classify the trophic strategy of the fish in San Sebastián Spring. To simplify the interpretation of this index, the standardized Levin niche width was used to express the result as a value ranging from 0 to 1, where 0 indicates a low niche breadth, and 1 signifies a wide niche breadth (Krebs, 1989).

The trophic diagram of Costello (1990) was used to graphically analyze the trophic strategy of *P. bimaculatus* by sex in the San Sebastián Spring, highlighting the relative importance of different dietary components. The abundance

of prey in the stomach content, expressed as a percentage of area, was plotted on the ordinate axis. Meanwhile, the frequency of occurrence in percentage was plotted on the abscissa axis. The diagram was divided into four quadrants, delineated by the 50 % thresholds for the percentage of area and the frequency of occurrence.

Reproductive biology: Fecundity was evaluated following the fecundity model of Schoenherr (1977) and Cabrera and Solano (1995) expressed by the following formula: (F = Ne + Nh), where Ne represents developed embryos and Nh corresponds to early developmental stages (fertilized eggs retained in the ovarian due the ovoviviparity reproduction of *P. bimaculatus*). per female. The gonadal stages were classified using the maturity scales following the criteria of Contreras-MacBeath and Ramírez-Espinoza (1996).

The gonadosomatic index (*GSI*) was calculated following the criteria of Zeyl et al. (2014), using the formula

$$GSI = \left(\frac{W_g}{W_t}\right) \times 100$$

where Wg = wet gonad weight, and Wt = total wet weight of the specimen.

The size at first maturity adjusted to 50 % of the population (L50) was determined. It was calculated using the standard length of the organisms found in the reproductive stage (stages IV, V, and VI). Through a logistic regression model using a sigmoidal model following the equation

$$M(L) = \frac{1}{1 + e^{-(aL+b)}}.$$

where M(L) represents the proportion of mature individuals at length L, and *a* and *b* are model parameters. Parameter a describes the slope of the maturity curve, reflecting the transition of individuals from immature to mature with increasing length, whereas b is related to the position of the curve along the length axis.

The sex ratio was obtained by counting the total number of females and males for each month and was determined with an x^2 test according to the criteria established by Sparre and Venema (1997).

The condition factor was evaluated according to the Fulton´s condition factor (K) (Froese, 2006): $K = 100\ (W/L^3)$, where W is the body wet weight (g), and L is the standard length (cm).

The type of growth was estimated with the formula , where W is the body weight, L is the standard length, b is the growth exponent or length weight, and a is a constant. Thus, a and b were determined by means of a linear regression (Froese, 2006).

Physicochemical water parameters: Water parameters were recorded before fish collection during each sampling event using a multiparameter probe (YSI EXO2; YSI Inc., Yellow Springs, OH, U.S.A). The following parameters were measured: dissolved oxygen (mg/L), total dissolved solids (TDS mg/L), conductivity (µS/cm), hydrogen potential (pH), and water temperature (°C).

Statistical analysis: We assessed the normality of univariate response variables evaluated using the Shapiro–Wilk test. As most variables did not meet normality assumptions, non-parametric tests were applied. Differences in trophic niche breadth and TL between sexes and seasons were assessed using Mann–Whitney tests. Differences among size classes and among sampling months were evaluated using Kruskal–Wallis tests, followed by Dunn's post hoc tests with Bonferroni correction for multiple comparisons.

Differences in diet composition according to sex, season, and size class were evaluated using a one-way PERMANOVA based on prey abundance data (mm^2), using a Bray–Curtis similarity matrix with 9 999 permutations.

We evaluated the relationship between female SL and fecundity (number of embryos) using Pearson's correlation coefficient. The gonadosomatic index (GSI) and Fulton's condition factor (K) were compared between sexes using Mann–Whitney tests and among months using Kruskal–Wallis tests with Dunn's post hoc comparisons. Departures from the expected

1:1 sex ratio were evaluated using chi-square tests (df = 1, α = 0.05). The length–weight relationship parameters were obtained from linear regression of log-transformed weight and length data.

Physicochemical parameters (dissolved oxygen, pH, temperature, conductivity, and total dissolved solids) were compared among sampling months using Kruskal–Wallis tests followed by Dunn's post hoc tests with Bonferroni correction for multiple comparisons.

All statistical analyses were performed in R version 4.4 (R Core Team, 2024) using the packages MVN (Korkmaz et al., 2014) and dunn.test (Dinno, 2026), except for the PERMANOVA analysis, which was conducted using PAST (version 4.17) (Hammer et al., 2001; Hammer, 2024),), and the estimation of size at first maturity (L50) and its confidence limits, which was performed using CurveExpert Basic version 2.2.3 (Hyams, 2020). The significance level was set at α = 0.05.

RESULTS

A total of n = 264 *P. bimaculatus* organisms were analyzed, 152 females and 112 males. For females, the size varied from a minimum of 15.71 mm and a maximum of 72.31 mm SL. While the smallest male measured 17.29 mm SL, and the largest was 50.92 mm SL.

Feeding habits: Out of the 264 specimens analyzed, 17 stomachs were empty, 109 were nearly empty, 91 were nearly full, and 47 were completely full. The diet of *P. bimaculatus* at San Sebastián Spring shows a diverse composition, consisting of a total of 17 food items (Table 1).

The diet did not differ significantly between seasons (PERMANOVA, pseudo-F = 2.26, df = 1, p = 0.08) or between size classes (PERMANOVA, pseudo-F = 1.6, df = 2, p = 0.1). However, there were significant differences observed between sexes (PERMANOVA, pseudo-F = 12.44, df = 1, p = 0.0001).

Table 1

Index of relative importance (% IRI) of each food item of *P. bimaculatus* by annual cycle, size class, and sex in the San Sebastián Spring, Mexico.

Food item	Annual	C-I	C-II	C-III	Females	Males
Hidracarinae	0	0	0.5	0	0	0
Aquatic insects	9	12	6	10	11	5
Algae	0	0	0	1	0	0
Spiders of Araneae	0	0	2	0	0	0
Chironomidae	1	0	0	1	0	0
Cladocerans	0	0	0	1	0	0
Copepods	0	0	1	0	0	0
Decapods	0	0	0	0	0	1
Unidentified Organic Matter	27	28	22	28	22	31
Unidentified eggs	3	7	2	1	0	29
Fish remains	3	3	1	4	2	5
Ants of Formicidae	5	3	15	8	7	1
Gastropods	0	0	0	1	1	0
Ostracods	0	0	0.5	0	0	0
Terrestrial Insects remains	29	26	29	23	33	12
Unidentified insect parts	23	21	21	21	24	15
Vegetable parts	0	0	0	1	0	1

C-I: Fish class size 1 including the females of 15.71-42.93 mm, and males of 17.29-28.50 mm. C-II: Fish class size II including the females of 42.94-57.62 mm, and males of 28.51-39.71 mm. C-III: Fish size class III including females > 57.63 mm, and males > 39.72 mm.

The food item exhibiting the highest IRI throughout the annual cycle was terrestrial insect remains, with an IRI of 29 %. Followed by detritus with an IRI of 27 %, while unidentified insect parts had an IRI of 23 % (Table 1). A trend was found for greater consumption of animal-origin resources by larger organisms, and the remains of terrestrial insects were found in a greater proportion in size II (IRI = 29 %). Ants from the Formicidae family were consumed greater in size II (IRI = 15 %) and size III (IRI = 8 %) (Table 1).

A higher consumption of terrestrial insect remains was observed in females (IRI = 33 %) compared to males (IRI = 12 %). Females consumed a greater quantity of aquatic insects (IRI = 11 %) than males (IRI = 5 %). This trend also extended to the consumption of Formicidae, with females exhibiting higher intake (IRI = 7 %) than males (IRI =1 %). Conversely, males demonstrated a higher consumption of detritus (IRI = 31 %) than females (IRI = 22 %) (Table 1).

The results of the standardized Levins niche breadth of *P. bimaculatus* indicated a narrow trophic niche (Bi = 0.20-0.44). No temporal (Mann-Whitney U test, W = 8417, p = 0.878) or ontogenetic (Kruskal–Wallis test, $\chi^2 = 5.89$, p = 0.053) differences were found. However, there were significant differences between the sexes (Mann-Whitney U test, W = 7097, df = 1, p = 0.021). Males exhibited a broader niche (Bi = 0.27), while females showed a narrower niche breadth (Bi = 0.21) (Table 2).

TL differed significantly between seasons (Mann-Whitney U test, W = 7097, p = 0.015) but not among size classes (Kruskal–Wallis test, $\chi^2 = 0.87$, df = 2, p = 0.646). However, we found differences between the sexes (Mann-Whitney U test, W =10786, p = 0.0001). The TL was highest in males (TL = 2.91 ± 0.36) in contrast with females (TL = 2.68 ± 0.39) (Table 2).

Costello diagrams show differences in trophic strategy between sexes. Females presented unidentified insect parts, detritus and terrestrial insect remains with high frequency and overall high abundance (Fig. 2A). In contrast, males showed a frequency of occurrence for detritus and unidentified insect remains that was equal to or greater than 50 %, but with a low overall abundance, representing less than 50 % of the percentage of area (Fig. 2B, SMF 2).

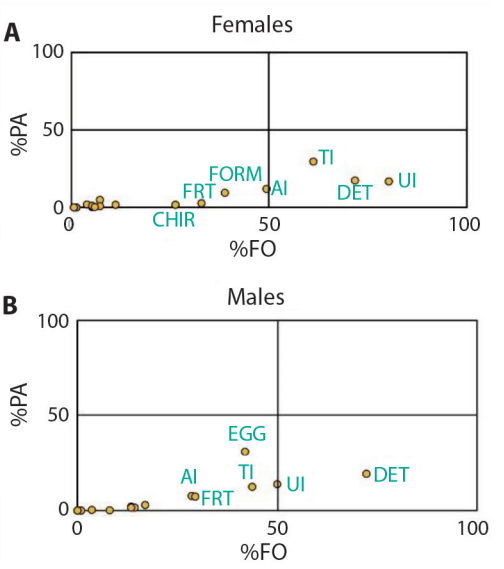


Fig. 2. Costello diagrams of *P. bimaculatus* from San Sebastián Spring, Mexico, showing variation in trophic strategy between **A.** Females and **B.** Males. Labels represent prey items with more than 25 % of % FO or % PA, as follows: UI, unidentified insect parts; DET, unidentified organic matter; TI, terrestrial insect remains; AI, aquatic insects; FORM, Formicidae (ants); FRT, fish remains; CHIR, Chironomidae; EGG, unidentified eggs.

Table 2

Niche breadth and trophic level of *P. bimaculatus* by annual cycle, size class, and sex in the San Sebastián Spring.

Index	Annual	C-I	C-II	C-III	Females	Males
Niche breadth (Levin´s Bi)	0.44	0.27	0.25	0.3	0.21	0.27
Trophic level (±SD)	2.89 ± 0.36	2.81 ± 0.36	2.92 ± 0.37	2.89 ± 0.38	2.68 ± 0.39	2.91 ± 0.36

C-I: Fish class size 1 including the females of 15.71-42.93 mm, and males of 17.29-28.50 mm. C-II: Fish class size II including the females of 42.94-57.62 mm, and males of 28.51-39.71 mm. C-III: Fish size class III including females > 57.63 mm, and males > 39.72 mm.

Reproductive biology: The sex ratio was balanced (1:1) in February (p = 0.572), April (p = 0.317), October (p = 0.500), and December (p = 0.680), with significant deviations in August (2:1, p = 0.003) and June (2:1, p = 0.046).

The fecundity model indicates that *P. bimaculatus* has a maximum of 82 eggs or embryos per female, with a minimum of 5 and an average of 21 eggs or embryos per female. It presents a significant correlation between the standard length of females versus the number of embryos (Pearson's coefficient, $R^2 = 0.74$, p = 0.001). In addition, the estimated size at first maturity(L50) indicates that females mature at 51.58 mm SL, while males mature at 34.42 mm SL, suggesting earlier reproductive onset in males.

The gonadosomatic index (GSI) showed significant differences between months (Kruskal–Wallis test, $\chi^2 = 7.92$, p = 0.019) (Fig. 3A). April showed the lowest mean fecundity and August, November and February with the highest mean fecundity, while the other months do not differ significantly (Fig. 3B), indicating that the reproductive peak is maintained throughout the year (Fig. 3A, Fig. 3B). Significant differences were observed between the sexes (Mann-Whitney U test, W = 13650, p = 2.2e-16), indicating that females have higher GSI values.

The length–weight relationship showed negative allometric growth in both sexes of *P. bimaculatus*. Females exhibited parameters of $a = 3.91 \times 10^{-5}$ and b = 2.806 ($R^2 = 0.97$), while

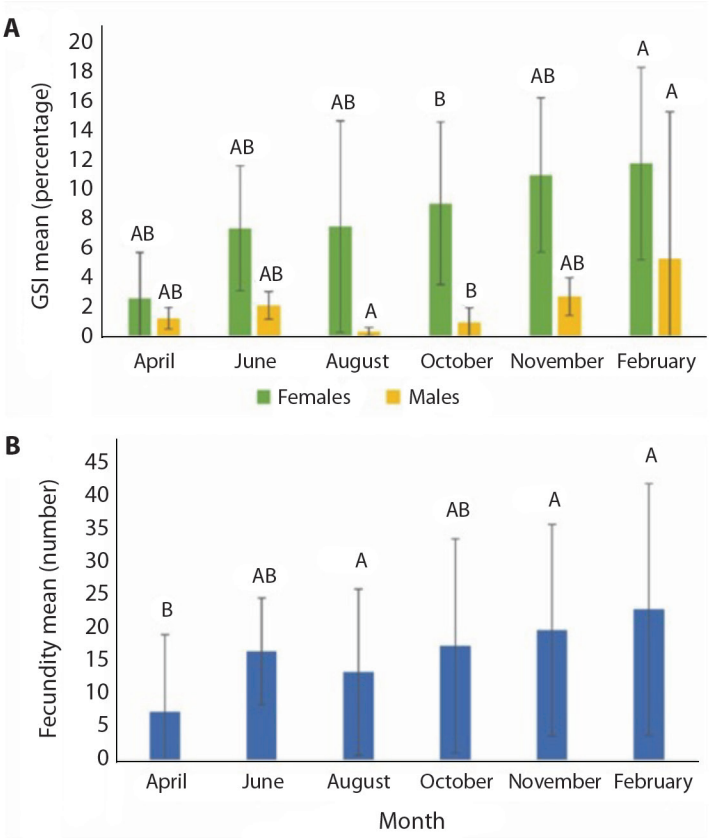


Fig. 3. Reproductive parameters of *Pseudoxiphophorus bimaculatus* from San Sebastián Spring, Mexico. **A.** Mean gonadosomatic index (GSI) by sex throughout the annual study cycle. **B.** Mean fecundity, expressed as the number of embryos per female, throughout the annual study cycle. The letters placed above the bars indicate the results of the post hoc Dunn test; different letters signify statistically significant differences.

males showed a = 1.46 × 10^{-4} and b = 2.398 (R^2 = 0.79). These results indicate that body weight increases at a lower rate than length in both sexes. The Fulton's condition factor indicates that the females are significantly more robust than males (w = 11404, p < 0.001). The Kruskal–Wallis test showed significant differences in the condition factor between months (χ^2 = 13.84, p = 0.017). The Dunn's posteriori tests indicate that April, June and August have the highest values of the Fulton´s condition index (Fig. 4).

The highest frequency of individuals in stage III and V were found throughout the year for both sexes, the lowest frequency of organisms was in stage I for both sexes. For June, no organisms in stage I and II were captured (Fig. 5).

Water parameters of the fish habitat: The dissolved oxygen varied from its lowest value of 6.14 ± 0.6 mg/L in August and peaking at 9.2 ± 0.55 mg/L in April. Water temperature reached a maximum value of 24.4 ± 0.52 °C in June and 24.87 ± 0.76 °C in August and a minimum of 17.8 ± 0.73 in February. The pH values indicated predominantly neutral water conditions (6.94-8.76), with a significantly higher value in December. Total dissolved solids were registered from a minimum of 103.1 ± 0.70 mg/L in February to a maximum of 162.05 ± 23.52 mg/L in October. Conductivity ranged from 93.63 ± 1.41 µS/cm in April to 120.7 ± 1.06 µS/cm in June.

All registered water parameters exhibited significant differences throughout the sampling year: Dissolved oxygen (Kruskal-Wallis test, χ^2 = 39.47, p < 0.0001), pH (Kruskal-Wallis test, χ2 = 37.99, p < 0.0001), total dissolved solids (Kruskal-Wallis test, χ^2 = 41.93, p < 0.0001), water temperature (Kruskal-Wallis test, χ^2 = 52.27, p < 0.0001) and conductivity (Kruskal-Wallis test, χ^2 = 44.72, p < 0.0001).

DISCUSSION

We describe the feeding habits and reproductive biology of Twospot livebearer *P. bimaculatus* in a small spring located in Central Mexico, providing new locality-specific ecological information for a non-native population in a conservation-relevant system. This fish species has been identified as invasive in aquatic systems and poses a potential risk to

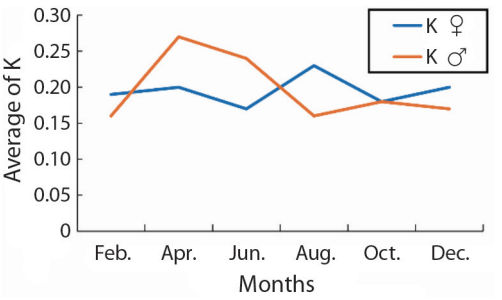


Fig. 4. Bimonthly average of Fulton factor condition (K) of *Pseudoxiphophorus bimaculatus*, from San Sebastián Spring, Mexico. Samples sizes per month: February: 50, April: 49, June: 16, August: 41, October: 55, December: 53.

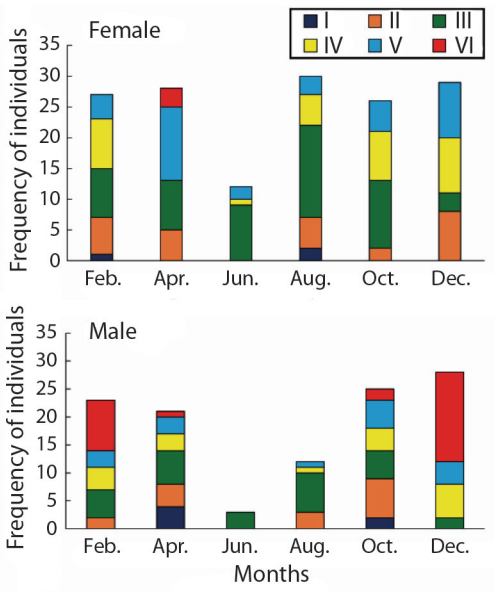


Fig. 5. Monthly variation in the frequency of gonadal stages of *Pseudoxiphophorus bimaculatus* from San Sebastián Spring, Mexico. **A.** Frequency of females recorded in each gonadal stage (I–VI). **B**. Frequency of males recorded in each gonadal stage (I–VI) during the same sampling months.

native ichthyofauna (Carbajal-Becerra et al., 2020; Mar-Silva et al., 2021; Suárez-Rodríguez et al., 2023). Our results indicate that this species exhibits an insectivorous diet and continuous reproductive activity throughout the year, traits associated with successful establishment in novel environments (Ramirez-García et al., 2018; Mar-Silva et al., 2021).

Feeding habits: Our results indicate that the diet of *P. bimaculatus* consists of 67 % terrestrial and aquatic insect remains, supporting its classification as carnivorous-insectivorous fish. This trophic pattern is consistent with previous studies conducted in non-native populations, where the species shifts from herbivory in its native range to increased consumption of animal-derived resources (Carbajal-Becerra et al., 2020; Mar-Silva et al., 2021; Trujillo-Jiménez & Toledo-Beto, 2007). Our results indicate that *P. bimaculatus* primarily feeds on terrestrial insects. Similar to our observations, Poecilids actively consume terrestrial insects attached to aquatic vegetation (Barroso-Magalhães et al., 2023; Ramírez-Herrejón et al., 2013). In the Teuchitlán River, *P. bimaculatus* demonstrates a high consumption rate of terrestrial insects, ranging from 49.8 % to 98.3 %. This indicates that the species may play a significant role in the allochthonous transfer of matter and energy into the water column (Mar-Silva et al., 2021), which may confer a potential advantage in food acquisition over other fish species and contribute to the energy flow within the ecosystem. Consequently, this species may influence the structure of the native food web, not only affecting fish assemblages but also by altering interactions with terrestrial insects, a dimension that remains poorly explored.

The standardized Levin's niche breadth values indicate a relatively narrow trophic niche, suggesting a tendency toward dietary specialization at the population level. However, the dominance of allochthonous resources, particularly terrestrial insects, together with variation in diet composition between sexes, indicates that the species can exploit locally available resources, indicating trophic flexibility and the ability to incorporate energy from surrounding terrestrial environments (Cucherousset & Olden, 2011; Nakano & Murakami, 2001). The higher consumption of insects observed in the species, and particularly for the females, may be associated with their energetic and nutritional requirements during reproduction (Boukal, 2014; Wootton, 1990). Insects represent a high-quality food resource rich in proteins and lipids, which are essential for oocyte development and embryo formation, thereby supporting reproductive investment in females (Halver & Hardy, 2002). This combination suggests that *P. bimaculatus* may exhibit a specialized diet composition with context-dependent resource use, rather than strict specialization.

In our study, we found that *P. bimaculatus* also consumes fish and gastropod eggs, a behavior previously reported in its non-native distribution (Carbajal-Becerra et al., 2020; Mar-Silva et al., 2021; Trujillo-Jiménez & Toledo-Beto, 2007). Observations indicate that this species preys on fish eggs and the remains of bony fish in various aquatic environments (Carbajal-Becerra et al., 2020). Additionally, it consumes a diverse array of animal matter, including insects from the Diptera and Hymenoptera orders, as well as the eggs and larvae of *Micropterus salmoides* (Trujillo-Jiménez & Toledo-Beto, 2007). In the Teuchitlán River, a highly human-impacted river near the San Sebastián Spring, *P. bimaculatus* has been found to consume unidentified fish remains, which constitute a secondary component of its diet (Mar-Silva et al., 2021). Our results also show that fish remains account for more than 5 % of the diet of *P. bimaculatus*. These results suggest that *P. bimaculatus* may exhibit opportunistic feeding behavior. This trophic plasticity has been previously reported for *P. bimaculatus* in non-native environments and may enhance its capacity to exploit diverse food resources (Carbajal-Becerra et al., 2020; Trujillo-Jiménez & Toledo-Beto, 2007).

Therefore, these results support the hypothesis that the species can present a trophic flexibility. This feeding strategy allows it to obtain food from the available resources

in the water body, a characteristic that can be advantageous when colonizing and invading new environments (Mar-Silva et al., 2021). Poeciliids, including the species under study, demonstrate a high trophic plasticity, reflecting their remarkable adaptability and opportunistic use of different resources (Gomez-Maldonado et al., 2023; Pollux & Reznick, 2011; Pyke, 2008; Ruehl & DeWitt, 2005).

Reproductive aspects: The reproductive traits observed for *P. bimaculatus* in San Sebastian Spring indicate a continuous reproductive strategy, with evidence of reproductive activity throughout the year. The GSI in *P. bimaculatus* females indicates that reproduction occurs throughout the year but with a reproductive peak in February. This pattern is consistent with previous reports for poeciliids in tropical and subtropical environments, where reproduction is often weakly seasonal or continuous (Gómez-Márquez et al., 1999; Ramírez-García et al., 2018). The same trend also occurred with the non-native poeciliids guppy *Poecilia reticulata* and Southern platyfish *X. maculatus* introduced in Brazil (Barroso-Magalhães et al., 2023).

The average fecundity of *P. bimaculatus* in the San Sebastián Spring is 21 developing embryos per female. This result supports the conclusions drawn from Gómez-Márquez et al. (1999) from "El Rodeo" dam in the state of Morelos; unlike Ramírez-García et al. (2018) which for the same species reports 16. In its native distribution, Olinger et al. (2016) determined an embryo count that ranged between 7 and 79 in the Cusuco National Park, Honduras. Schoenherr (1977) points out that fecundity differences are influenced by several factors, such as nutrition, predation, water temperature, and photoperiod. These factors can affect the reproductive period, the number of oocytes produced by each female, the size of the offspring, and the timing of their birth.

Our results reveal a difference in size at first maturity between the sexes, reaching earlier in males than in females. This is consistent with what was reported in other studies carried out with poeciliids, where the males exhibit earlier maturation than females, growth ceases when the gonopodium is completely formed, and males do not live long after reaching maturity (Snelson, 1984). On the other hand, females in *P. bimaculatus* have a long maturation period and high growth rates (Gómez-Márquez et al., 1999). In this way, we can infer that in the San Sebastián Spring, the reproduction of males occurs at an early age, allowing them to find sexually mature females and enhance the production of offspring. Our results of the Pearson correlations indicate that larger females have more embryos (relationship between standard length and fecundity $R^2 = 0.75$, $p < 0.0001$); The positive relationship between female size and fecundity suggests that larger females contribute disproportionately to reproductive output, a common pattern in livebearing fishes (Contreras-MacBeath & Ramírez-Espinoza, 1996).

The length–weight relationship observed in *P. bimaculatus* indicates negative allometric growth in both sexes, suggesting that individuals increase in length at a faster rate than in weight (Froese, 2006; Karachle & Stergiou, 2012). This growth pattern has been previously reported in the species and may be associated with life-history strategies that favor reproductive output (Gómez-Márquez et al., 1999; Ramírez-García et al., 2018). In this context, the lower weight gain relative to length could reflect an energy allocation strategy in which resources are directed toward reproduction rather than somatic mass. Therefore, our results are consistent with previous findings and support the idea that the allometric growth pattern of *P. bimaculatus* may contribute to its reproductive potential and ecological success.

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idea that the allometric growth pattern of *P. bimaculatus* could be related to its reproductive potential and ecological success (Winemiller & Rose, 1992).

We found that the reproduction of *P. bimaculatus* in San Sebastián Spring occurs continuously throughout the year. The observed gonadal stages (stages III and V) indicate that mature individuals are present year-round. This pattern is characteristic of a fish species with high reproductive potential, likely an r-strategist (MacArthur, 1962). Additionally, the fish-sex ratio aligns with this reproductive strategy. In June, we found only mature individuals, and the sex ratio for June and August showed a higher number of females than males (2:1). This female-biased sex ratio enhances reproductive success, ensuring population propagation and invasion potential (Barroso-Magalhães & Jacobi, 2017; Barroso-Magalhães et al., 2023; Ramírez-García et al., 2018). Meanwhile, the condition factor suggests that individuals are in robust condition, particularly during periods when mature organisms were observed (June) and when the proportion of females was the highest (June and August), which is to be expected in viviparous species, where the embryos influence the condition factor (Pollux & Reznick, 2011; Wourms, 1981).

This is mediated by the differential energy distribution of individuals during the reproduction process, driving females to gain weight and increase in size and males to grow and express their secondary sexual characters, allowing a temporary synchrony that maximizes the reproductive events.

Therefore, our findings on the reproductive biology of *P. bimaculatus*—such as the higher number of females, the presence of individuals at all gonadal stages throughout the annual cycle, and the dominance of sexually mature, robust individuals capable of reproduction—are consistent with observations from other sites outside its native distribution range (Ramírez-García et al., 2018). This suggests that *P. bimaculatus* has successfully established a reproducing population in the San Sebastián Spring, as indicated by the continuous reproductive activity and the presence of mature individuals throughout the year (Lockwood et al., 2013). Accordingly, *P. bimaculatus* exhibits biological traits that may facilitate its establishment and persistence as a non-native species in San Sebastián and in other locations further from its native range.

Environmental water characteristics of San Sebastián Spring: *P. bimaculatus* reproduces in non-native environments within a temperature range of 23.6-29.2 °C and a pH of 6.5-8.3, in well-oxygenated, moderately hard water with high total alkalinity (Gómez-Márquez et al., 1999). These conditions are comparable to those reported by Ramírez-García et al. (2018), which found that the species reproduces in temperatures ranging from 24.3-27.1 °C, with a pH of 6.7-7.5, in hard waters with high alkalinity and dissolved solids. In contrast, Olinger et al. (2016) reported that in its native range, *P. bimaculatus* reproduces at lower temperatures, between 16.9 °C and 25.1 °C, with a pH ranging from 5.0 to 7.5. In these native habitats, oxygen levels vary between 2.0 mg/L and 10.2 mg/L at altitudes ranging from 50 to 1 600 m.a.s.l. In San Sebastián Spring, water temperatures range from 17 to 24.87 °C, with a predominantly neutral pH.

The data about temperature in native and non-native distribution indicated that *P. bimaculatus* exhibits tolerance for temporal variability in environmental conditions. Our temperature range result is similar to the natural fluctuations in its native habitats. This demonstrates significant plasticity for survival and successful reproduction across varying environmental conditions—an essential characteristic of invasive species (Lockwood et al., 2013). The results of the reproductive biology of *P. bimaculatus* in San Sebastián Spring showed that the fish can reproduce continuously throughout the year, which would cause to present a long reproductive period in a similar way as it happens in other water bodies (Gómez-Márquez et al., 1999; Ramírez-García et al., 2018). However, data suggest that environmental conditions of San Sebastián are not constant over the year

and may be influenced by seasonal changes, associated to precipitation and runoff processes. This illustrates the high capacity of the species to tolerate, survive, and reproduce in changing conditions, which may confer an advantage over more sensitive species.

Our present study provides new ecological information on the feeding habits and reproductive biology of *P. bimaculatus* in San Sebastián Spring, an essential freshwater system for conserving native and endemic species of Central Mexico (Domínguez-Domínguez et al., 2008). Our results indicate that this species exhibits a predominantly insectivorous diet and continuous reproductive activity throughout the year, suggesting a capacity to exploit locally available resources and maintain stable populations. The studied aquatic system showed temporal variability in water parameters. However, *P. bimaculatus* presented its characteristics of trophic and reproductive biology under variable environmental conditions, reinforcing the idea that it is a species with great flexibility in expressing its life history.

These trophic and reproductive traits are commonly associated with successful establishment in non-native environments and may facilitate the persistence of *P. bimaculatus*. However, the present study does not directly assess interactions with native invertebrates and fish species or their potential ecological consequences. Therefore, further research integrating community-level approaches will be necessary to evaluate the extent to which this species may influence native biodiversity in the San Sebastián Spring.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

See supplementary material
A43v74n1-SUPPL 1

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