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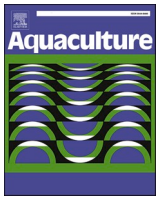

Effective control of *Neobenedenia girellae* infestation by an optimized oral protocol in greater amberjack juveniles (*Seriola dumerili*)☆

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ABSTRACT

Infestations by *Neobenedenia girellae* constitute significant challenges to the culture of greater amberjack (*Seriola dumerili*), a promising species for aquaculture diversification. The impact of these infestations is further amplified by global ocean warming, underscoring the urgent need for effective prevention and control measures. This study assessed the efficacy of different praziquantel (PZQ) treatments on growth performance, muscle kinetic profiles (Experiment 1), and their ability to control *N. girellae* infestations in *S. dumerili* juveniles reared in an open seawater system (Experiment 2). In Experiment 1, six treatments were tested: T1 (Control), T2-PZQ 2.25 %-1 (450 mg PZQ/kg live weight, single dose), T3-PZQ 1.125 %-2 (225 mg PZQ/kg live weight, 2 days), T4-PZQ 0.75 %-3 (150 mg PZQ/kg live weight, 3 days), T5-PZQ 0.75 %-2 (150 mg PZQ/kg live weight, 2 days), and T6-PZQ 0.75 %-1 (150 mg PZQ/kg live weight, single dose). In Experiment 2, protocols T1–T4 were evaluated. Results indicated that growth performance was unaffected by PZQ treatments, and muscle PZQ concentrations returned to baseline within 48 h post-administration. Protocols T3–1.125 %-2 and T4–0.75 %-3 exhibited the highest efficacy against *N. girellae* adults, achieving 91 % and 99.8 % parasite reduction, respectively. The protocol involving 150 mg PZQ/kg administered over three consecutive days proved to be the most effective for managing *N. girellae* infestations, without compromising fish growth and with minimal muscle residue persistence.

1. Introduction

The greater amberjack (*Seriola dumerili*) is a cosmopolitan marine teleost distributed in tropical and subtropical areas (45°N–28°S) of the Atlantic and Indo-Pacific Oceans (FAO, 2024). It is a promising species for aquaculture diversification due to its rapid growth, excellent flesh quality, and high consumer acceptance (Mylonas et al., 2017; Roo et al., 2019), with prices reaching up to 50 USD (United States Dollars) per kg in the Japanese market. Additionally, the greater amberjack is one of the largest species of *Seriola*, reaching up to 6 Kg of weight in just 2.5 years (FAO, 2024).

However, one of the main challenges to the further expansion of *Seriola*'s aquaculture is the high incidence of external parasites, particularly neodermatan species during the on-growing phase (Hirazawa et al., 2016; Fernández Montero, 2020). Specifically, the greater amberjack is highly susceptible to *Neobenedenia girellae*, a monopisthocotylean parasite that infects the fish's skin, feeding on epithelial cells and causing haemorrhagic and ulcerative lesions, epidermal hydropic degeneration, thickening, and secondary infections. These infections lead to growth impairments and high mortality rates when

Abbreviations: ACN, acetronile; ESI, electrospray ionisation; EU, European Union; FCR, feed conversion ratio; FI, feed intake; FO, fish oil; LC-MS, water of mass spectrometry; LOQ, quantification limit; MeOH, methanol; MS/MS, triple quadrupole tandem mass spectrometer; NMP, N-metil-pirrilodone; PZQ, praziquantel; RAS, recirculating aquaculture systems; RF, radio frecuency; S/N, signal-to-noise ratio; SGR, specific growth ratio; T1, control treatment; T2, treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose; UHPLC, Ultra-High Performance Liquid Chromatography; USD, United States Dollars; USE, ultrasound assisted extraction; *v*/v, volume per volume; WG, weight gain.

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parasitism is severe (Hirayama et al., 2009; Hirazawa et al., 2016; Fernández Montero, 2020).

Preventive treatments, such as freshwater baths lasting 2–5 min, are commonly used to control parasite levels. However, these practices involve additional labour and stress for the fish (Hirazawa et al., 2013) and are not always feasible depending on the culture conditions. Moreover, although dilute formalin preparations are the most used agents against fish ectoparasites in aquaculture (Bader et al., 2019; Rigos et al., 2024) formalin baths may not be suitable as they can harm fish tissues, reduce dissolved oxygen in the water, and damage essential microbes in biofilters, particularly in recirculating aquaculture systems (RAS) (Bader et al., 2019).

Praziquantel (PZQ) is a synthetic drug effective against a broad range of endo and ectoparasites and is used in both human and veterinary medicine (Kogiannou et al., 2021). The drug disrupts the parasite's tegument, causing spastic muscular paralysis by binding Ca^{2+} channels and disrupting Ca^{2+} homeostasis through changes in membrane permeability. In mammals, this compound has a wide safety range (reviewed by Bader et al., 2019).

PZQ has been tested in other *Seriola* species and is authorized for use as antiparasitic in countries such as Japan, Australia or the Philippines. However, in Spain or any other European country, it is not legally authorized or registered for commercial use in marine fish farms (Rigos et al., 2024), although it is approved for humans, farm animals and pets. In fish, PZQ can be used 'off-label', with a standard withdrawal time of 500-degree days, and an adopted maximum residue level of zero to secure consumer safety (Rigos et al., 2021a, 2021b).

Several studies have tested the use of PZQ against fish parasites, both through bath and oral administration, with doses ranging from 7.5 to 800 mg/kg/day (reviewed by Bader et al., 2019). Specifically, PZQ administered at 150 mg/kg for 3 days showed high efficacy (>80 %) against the gill monopisthocotylean *Zeuxapta seriolae* in greater amberjack (Rigos et al., 2021a) and *N. girellae* in chub mackerel (*Scomber japonicus*) (Yamamoto et al., 2011). However, specific therapeutic regimens for *N. girellae* in greater amberjack have only been tested at low doses over extended periods (Kogiannou et al., 2021; Rigos et al., 2021b).

Furthermore, global ocean warming may lead to an increase in the frequency and severity of parasitic infestations, among other reasons, by enhancing rates of parasite growth and development (Macnab and Barber, 2012). Therefore, effective prevention and control protocols are urgently needed to increase the resilience of marine aquaculture.

Based on the above, this study aimed to evaluate different oral praziquantel (PZQ) protocols to define the most effective treatment for controlling and treating *Neobenedenia girellae* infestations in greater amberjack (*Seriola dumerili*) juveniles. For this purpose, the research provides insights into the pharmacokinetics of PZQ in muscle tissue, assesses the life cycle dynamics of *N. girellae* under ocean warming conditions, and examines key performance indicators such as growth, feed utilization, and overall fish health under different treatment protocols. The results contribute to providing the scientific support needed for the authorization of PZQ use by the aquaculture sector in Europe and other regions worldwide.

2. Material and methods

2.1. Experimental design and rearing conditions

This study was divided into two experiments:

2.1.1. Experiment 1

This experiment aimed to determine the effects of different praziquantel (PZQ) administration protocols on the growth of *S. dumerili* juveniles compared with a control, as well as to assess the drug depletion profile in muscle at different sampling points. In April 2023, 210 greater amberjack juveniles obtained from induced spawnings of acclimated broodstock were distributed in 18 tanks (300 L each, 12 fish per tank, initial weight around 120 g, rearing density below 7 kg/m^3). Fish were maintained in an open seawater system in which the natural seawater just overpassed a sedimentation tank prior to the arrival at the experimental tanks, which were kept under natural temperature conditions and controlled photoperiod (12 h light, 12 h dark). Water temperature and oxygen levels were monitored daily with a digital probe (OxyGuard, Handy Polaris, Acuitec S.L. Gipuzkoa, Spain). During the experimental period (April–May), temperatures ranged from 20.5 to 21.5 °C and oxygen concentration remained at 6 mg/L.

Six treatments were administered every 2 weeks in triplicated tanks: T1-Control (commercial diet R3, Skretting, Burgos, Spain), T2-PZQ 2.25 %-1 (commercial diet +450 mg PZQ/kg live weight, equal to 2.25 % of PZQ in feed, single dose), T3-PZQ 1.125 %-2 (commercial diet +225 mg PZQ/kg live weight, equal to 1.125 % of PZQ in feed, 2 consecutive days); T4-PZQ 0.75 %-3 (commercial diet +150 mg PZQ/kg live weight, equal to 0.75 % of PZQ in feed, 3 consecutive days); T5-PZQ 0.75 %-2 (commercial diet +150 mg PZQ/kg live weight, equal to 0.75 % of PZQ in feed, 2 consecutive days); T6-PZQ 0.75 %-1 (commercial diet +150 mg PZQ/kg live weight, equal to 0.75 % of PZQ in feed, single dose).

The cumulative dose for treatments T1 to T4 was 450 mg of PZQ/kg live weight, for T5 it was 300 mg PZQ/kg, and for T6 it was 150 mg PZQ/kg. Due to size variability, treatments T1 to T4 were conducted with fish weighing between 130 and 216 g (168 ± 20 g) while treatments T5 and T6 involved fish weighing between 85 and 141 g (110 ± 15 g).

To prepare the experimental diets, the specific PZQ dose (≥98 % purity, Sigma Aldrich; Spain) was mixed with 10 g of fish oil (FO) per kilogram of the experimental diet. The mixture was combined with the commercial fed (R3, Skretting, Burgos, Spain). The resulting diets were stored at 4 °C until use, and samples of each experimental diet were frozen at −80 °C until PZQ analyses were conducted.

Feeding was done manually to apparent satiation, twice daily (9:00 and 14:00 h). Uneaten feed was collected, dried for 24 h at 100 °C and weighted to adjust feeding amounts. Experimental diets were administered every two weeks according to the specific pattern for each treatment (T2 and T6 as single doses, T3 and T5 over two consecutive days and T4 over three consecutive days). Between treatments, all the fish were fed the control diet.

Weight, length and ectoparasites presence were monitored every two weeks. To check for ectoparasites, fish were bathed in freshwater for 4 min (Hirazawa et al., 2016) and the water was filtered through a 125 μm net to detect parasites using a stereoscope. Parasite monitoring results were not considered in this first study, as natural infestation did not occur during the experiment.

The final sampling was conducted after 50 days of experimentation, for which three fish per treatment were sacrificed in the early morning via clove oil overdose at 24, 48 and 72 h after treatment initiation (Table 1). Fish weight and length were measured, and, for PZQ determination, 4–5 g samples were collected without skin from the dorsal musculature of the fish right side (taking as reference the the muscle just below the dorsal fin). The muscle samples were stored at −80 °C until analyzed.

2.1.2. Experiment 2

This experiment aimed to evaluate the effectiveness of the experimental protocols defined in experiment 1 for controlling *N. girellae* infestation in an open seawater system. In July 2023, the presence of *N. girellae* in the *S. dumerili* stock was confirmed through routine monitoring described in Experiment 1. In this moment, 40 greater amberjack juveniles (249 ± 51 g initial weight) were selected and anaesthetized with clove oil-ethanol (1:1) and individually weighted, measured, microchipped and distributed into eight cleaned 1000 L tanks in an open seawater system. Before distribution, the fish were given a 4-min freshwater bath (Hirazawa et al., 2016) to ensure the absence of parasites at the start of the trial.

Table 1
Sampling protocol to obtain *Seriola dumerili* muscle samples at different moments after the treatments (Experiment 1, $n = 3$ fish per treatment).

	Sunday	Monday	Tuesday	Wednesday	Thursday
T1-Control	Starvation	T1-Control	T1-Control	T1-Control	T1-Control
T2-PZQ 2.25 %-1	Starvation	T2-PZQ 2.25 %-1	T1-Control	T1-Control	T1-Control
T3-PZQ 1.125 %-2	Starvation	T3-PZQ 1.125 %-2	T3-PZQ 1.125 %-2	T1-Control	T1-Control
T4-PZQ 0.75 %-3	Starvation	T4-PZQ 0.75 %-3	T4-PZQ 0.75 %-3	T4-PZQ 0.75 %-3	T1-Control
T5-PZQ 0.75 %-2	Starvation	T5-PZQ 0.75 %-2	T5-PZQ 0.75 %-2	T1-Control	T1-Control
T6-PZQ 0.75 %-1	Starvation	T6-PZQ 0.75 %-1	T1-Control	T1-Control	T1-Control
Sampling moment			**24 h**	**48 h**	**72 h**

Abbreviations: PZQ, praziquantel; T1-Control; control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

The following day, 12.5 cm^2 threads of polyester obtained from two infected donor tanks containing an average of 87 *N.girellae* eggs/thread, were evenly distributed in the experimental tanks to ensure controlled exposure to the target parasite.

After this, protocols 1 to 4 described in experiment 1 (T1-Control: commercial diet R3, Skretting, Burgos, Spain; T2-PZQ 2.25 %-1: commercial diet +450 mg PZQ/kg live weight, single dose; T3-PZQ 1.125 %-2: 225 mg PZQ/kg live weight for 2 consecutive days; T4-PZQ 0.75 %-3: 150 mg PZQ/kg live weight for 3 consecutive days), were applied weekly in duplicate. Feeding was performed manually twice per day to apparent satiation (2 tanks and 10 fish individually monitored per treatment). Water temperature and oxygen were measured daily using a digital probe (OxyGuard, Handy Polaris, Acuitec S.L. Gipuzkoa, Spain), with temperatures ranging from 21.5 to 23.8 °C and oxygen levels maintained at 6 mg/L.

In addition, 61.5 cm^2 white polyester threads were placed daily in each tank to monitor the presence of *N.girellae* eggs after 24 h under a stereoscope. Every two weeks, the fish were also anaesthetized with a clove oil-ethanol solution (1:1) and given freshwater baths to evaluate the presence of adult *N. girellae*. Each fish was bathed individually in separated containers for 4 min, after which the water was filtered through a 125 μm net and examined under a stereoscope to count the number of *N.girellae* adults.

The experiment lasted for 27 days (July–August 2023). The efficacy of the treatments was calculated using the following formula:

Efficacy (%) = ((Number of *N.girellae* adults in T1-Control − Number of *N.girellae* adults in experimental protocol) / Number of *N.girellae* adults in T1-Control)*100.

All experimental procedures involving animals were conducted in accordance with the European Union Directive (2010/63/EU) on animal welfare for scientific purposes, at the facilities of the GIA-Ecoaqua group, University of Las Palmas de Gran Canaria (ULPGC). The experimental protocol was approved by the Bioethical Committee of the ULPGC (number of protocol OEBA_ULPGC_18/2023).

2.2. Growth and feed utilization

At the end of experiment 1, growth and feed utilization parameters were calculated using the following equations:

$$\text{Weight Gain (WG) (\%)} = 100^{*}(\text{Final weight (g)} - \text{Initial weight (g)}) / \text{Initial weight (g)}.$$

$$\text{Specific Growth rate (SGR) (\%/day)} = ((\ln Wf - \ln Wi)/\text{Days of experiment}) \times 100.$$

Where:
lnWf: Neperian logarithm of the final weight.
lnWi: Neperian logarithm of the initial weight.

$$\text{Feed Intake (FI) (\%)} = (\text{Estimated feed consumption (g)} / \text{Average fish weight (g)})/\text{Days} \times 100.$$

$$\text{Feed Conversion Ratio (FCR)} = \text{Estimated feed consumption (g)} / \text{Weight gain (g)}.$$

2.3. Analysis of praziquantel levels in diets and muscle

2.3.1. Reagents and consumables

Acetonitrile (ACN) and water of mass spectrometry (MS) grade, used for the mobile phase, as well as methanol (MeOH), ACN, acetone, hexane and formic acid (purity <99 %) for extraction and cleaning of the chromatographic system, were supplied by Panreac Química (Barcelona, Spain). The 0.2 μm polyethylene terephthalate (PET) syringe filters were purchased from Macherey-Nagel (Dueren, Germany). The stock solution was prepared in MeOH at 100 $mg{\cdot}L^{-1}$ and stored in capped glass bottles at −20 °C in the dark.

2.3.2. Instrumentation

Praziquantel (PZQ) was determined using an ACQUITY Ultra-High Performance Liquid Chromatography (UHPLC) system (Waters Chromatography, Barcelona, Spain) equipped with a binary solvent manager for analyte elution, a thermostatically controlled 2777 autosampler and a column for temperature control. The system was coupled to a triple quadrupole tandem mass spectrometer (MS/MS) detector with electrospray ionisation (ESI) and controlled by MassLynx mass spectrometry software.

2.3.3. Chromatographic and detection conditions

PZQ was identified with a Luna C_{18} column (150 × 4.6 mm, 5 μm particle size) from Phenomenex (Madrid, Spain) with a column temperature of 35 °C and a flow rate of 0.3 $mL{\cdot}min^{-1}$. The isocratic mobile phase consisted of LC-MS grade water (A) (65 %) and acetonitrile (B) (35 %), both containing 0.1 % (*v*/v) formic acid. For mass spectrometric detection, the ESI parameters were set as follows: capillary voltage at 3 kV; cone voltage at 30 V; extractor voltage at 3 V; radio frequency (RF) lens voltage at 1 V; desolvation temperature at 140 °C and source temperature at 450 °C; desolvation gas flow rate at 500 $L{\cdot}hr^{-1}$ and cone gas flow rate at 50 $L{\cdot}hr^{-1}$. Nitrogen was used as the desolvation gas and

Table 2
Experimental design (2^4) used for optimization of USE procedure.

Experiment number	Time (min)	Sample weight (mg)	Extraction solvent	Volume of solvent (mL)
1	10	300	ACN	5
2	20	300	MeOH	5
3	10	100	MeOH	5
4	20	100	ACN	5
5	10	100	MeOH	10
6	10	300	ACN	10
7	10	100	ACN	10
8	20	100	MeOH	10
9	10	300	MeOH	10
10	20	300	MeOH	10
11	20	100	ACN	10
12	20	300	ACN	10
13	10	100	ACN	5
14	20	300	ACN	5
15	20	100	MeOH	5
16	10	300	MeOH	5

Abbreviations: ACN, acetronile; MeOH, methanol; USE, ultrasound assisted extraction.

argon as the collision gas. The precursor ion (m/z) was set at 313.2, while fragment ions were selected at 203.1 (capillary energy at 20 V) and 213.04 (capillary energy at 40 V).

2.3.4. Optimization of sample extraction

An ultrasound-assisted extraction (USE) procedure for PZQ determination was optimized by testing variables such as time, sample weight, extractant and volume. Feed samples were spiked to a final concentration of 500 μg/L in the extract, and 16 randomized experiments based on a 2^4 design (Table 2) were conducted. Time duration of 10 and 20 min, sample weights of 100 and 300 mg and extractant volumes of 5 and 10 mL for both acetronile (ACN) and methanol (MeOH) were tested. Minitab version 22.1 (Minitab, LLC, State College, PA, USA) was used to analyze the influence and relationships between variables. The best results were obtained using 10 min, 300 mg of sample and 5 mL of MeOH yielding an extraction efficiency of 92.12 % of PZQ. The method was then validated with fish samples, achieving a recovery of 85.35 % slightly lower value due to the complexity of the fish matrix, though still adequate for PZQ extraction.

2.3.5. Quality control

After optimizing the method, parameters such as linearity, limit of quantification (LOQ), and extraction efficiency were evaluated. A calibration curve was prepared with eight points ranging from 1 to 500 μg/L, achieving a R^2 of 0.9994. The LOQ was determined based on the signal-to-noise ratio (S/N) of individual compound responses with an instrumental LOQ of 0.093 μg/L, and a method LOQ of 1.78 ng/g. Extraction efficiencies (recoveries) were assessed by adding a known amount of standard into 300 mg of sample to achieve a final concentration of 500 μg/L in the final theoretical extract. As detailed in the optimization section, recovery was 92.12 % for feed samples and 85.35 % for fish samples.

2.4. Neobenedenia girellae life cycle monitorization

Once the presence of parasites was confirmed and prior to the beginning of the experimental protocols described in Experiment 2, live adult parasites ($n = 10$) were carefully collected from the fish skin with a scalpel and placed individually in plastic plates (10 cm diameter) filled with seawater. All the threads placed in the experimental tanks were replaced by clean ones every 24 h. The eggs attached to the threads during this period were counted under the stereoscope. Eggs from the untreated tanks were placed in 300 mL beakers containing seawater, while those from the PZQ-treated tanks were discarded. Both the plates and the beakers were maintained under natural photoperiod and temperature conditions, being the water temperature recorded daily before the routine replacement with fresh seawater. Both the time to hatching of the eggs in the plates and the beakers were tracked through daily inspection of the eggs under the stereoscope. Measurements of alive adults (total length, $n = 10$) and eggs (total surface area considering both sides and perimeter, $n = 30$) were conducted using Leica Application Suite software (Leica Microsystems Ltd., Heerbrugg, Switzerland).

The following formulas were utilized:

Time to hatching (plates) = Time (days) from spawning to hatching.

Estimated time to hatching (beakers)
= Time (days) from attachment in the polyester threads to hatching.

Adult survival without a host = The number of days from removal from the fish skin until death.

Oncomiracidia survival without a host
= The number of days from hatching until death.

Daily fecundity = The number of eggs produced by each adult, counted 24 h after being placed in the plates.

2.5. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistic 20 (New York, NY, USA). After testing for normality and homoscedasticity, variance analyses were conducted using one-way ANOVA and means were compared with Tukey posthoc test. Significant differences were considered when $P < 0.05$. The experimental unit in the experiment 1 was the tank (triplicated for each treatment), while the experimental unit considered for experiment 2 was the individual (10 microchipped fish per treatment). Due to the two fish size ranges used in experiment 1, the statistical analyses of growth parameters were performed separately for treatments T1 to T4 and between T5 and T6.

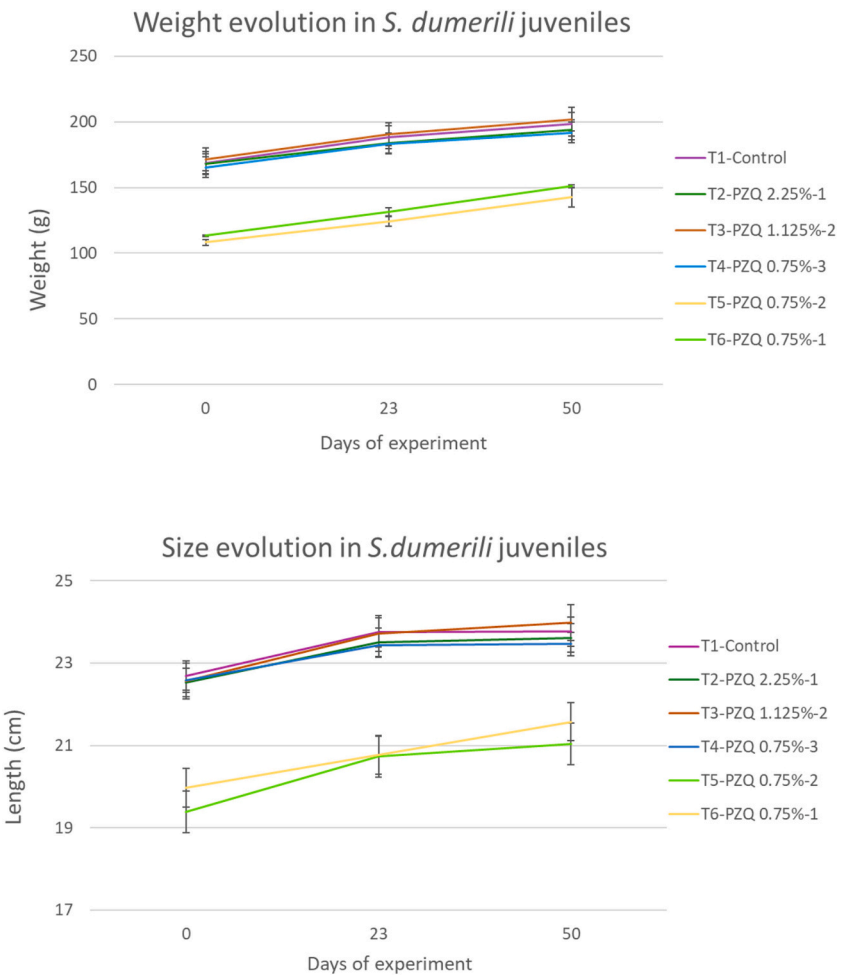

Fig. 1. Weight and length evolution in *S.dumerili* juveniles along the experimental period (Experiment 1, $n = 3$ tanks per treatment with 12 fish/tank). Lines without superscripts indicate the absence of significant differences ($P \geq 0.05$) (comparisons between T1-T4 and T5-T6). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel; T1-Control, control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

Table 3
Growth and feed utilization parameters for *S.dumerili* juveniles along the experimental period (Experiment 1, n = 3 tanks per treatment with 12 fish/tank).

	SGR	Weight gain (%)	FCR	Feed intake (%)
T1-Control	0.60 ± 0.08	17.66 ± 2.56	4.09 ± 0.47	1.73 ± 0.10
T2-PZQ 2.25 %-1	0.53 ± 0.11	15.53 ± 3.57	4.60 ± 1.19	1.69 ± 0.21
T3-PZQ 1.125 %-2	0.60 ± 0.10	17.68 ± 3.32	4.11 ± 0.60	1.74 ± 0.22
T4-PZQ 0.75 %-3	0.55 ± 0.08	15.98 ± 2.38	3.97 ± 0.55	1.53 ± 0.02
T5-PZQ 0.75 %-2	1.02 ± 0.15	31.82 ± 5.22	2.76 ± 0.40	1.98 ± 0.30
T6-PZQ 0.75 %-1	1.06 ± 0.05	33.21 ± 1.67	2.61 ± 0.32	1.95 ± 0.24

Table 4
Levels of praziquantel (PZQ) in the experimental diets (g/Kg).

Diet	PZQ levels (g/Kg diet)	
	Theorical	Measured
Control (T1)	0	–
PZQ 2.25 %-1 (T2)	22.5	12.43
PZQ 1.125 %-2 (T3)	11.25	6.48
PZQ 0.75 %-3 (T4, T5, T6)	7.5	3.61

Abbreviations: Control (T1), control diet provided in treatment 1; PZQ 2.25 %-1 (T2); commercial diet +450 mg PZQ/kg live weight provided in treatment 2; PZQ 1.125 %-2 (T3), commercial diet +225 mg PZQ/kg live weight provided in treatment 3; PZQ 0.75 %-3 (T4, T5, T6) commercial diet +150 mg PZQ/kg provided in treatment 4, 5 and 6.

3. Results

3.1. Growth and feed utilization (Experiment 1)

The various treatments did not result in significant differences in weight or size across the different sampling points (Fig. 1). Similarly, no differences were observed in the specific growth rate (SGR), weight gain percentage (WG), feed conversion ratio (FCR) or daily feed intake (FI) (Table 3).

Columns without superscripts indicate de absence of significant differences ($P \geq 0.05$) (comparisons between T1-T4 and T5-T6). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel; T1-Control, control treatment; T2-PZQ 2.25 %-1; treatment

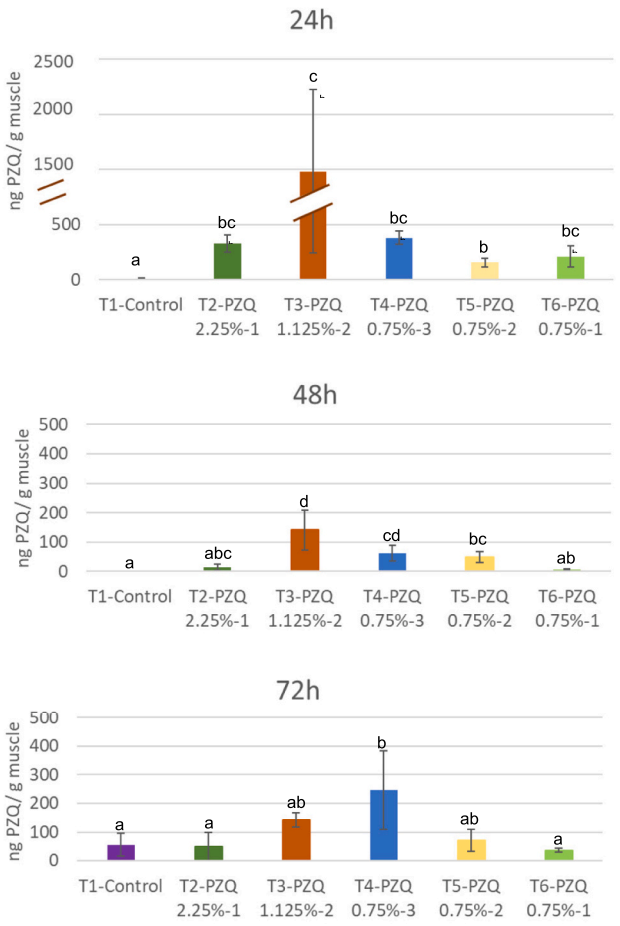

Fig. 2. Praziquantel muscle levels (ng/g muscle) at different sampling points (Experiment 1, $n = 3$ tanks per treatment with 12 fish/tank). Columns with different superscripts indicate significant differences among groups ($P < 0.05$). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel, T1-Control, control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

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3.2. Analysis of praziquantel levels in diets and muscle (Experiment 1)

The PZQ levels in the experimental diets, measured using Ultra-High Performance Liquid Chromatography coupled with a triple quadrupole tandem mass spectrometer detector (UHPLC-MS/MS), are presented in Table 4.

The levels of PZQ in muscle varied significantly depending on the treatment and the sampling time ($P < 0.05$). From 24 h to 48–72 h post-treatment, there was a significant reduction of 78 % in the PZQ levels in muscle ($P < 0.05$), with no significant differences between the 48 and 72-h sampling points ($P > 0.05$).

Regarding treatment comparisons (Fig. 2), at the 24-h sampling point, PZQ levels in the muscle were statistically equivalent across treatments, except for a difference between T3 (PZQ 1.125 %-2) and T5 (PZQ 0.75 %-2).

At the 48-h sampling point, T3 (PZQ 1.125 %-2) had the highest PZQ content in muscle, followed by T4 (PZQ 0.75 %-3), while single dose treatments (T2 and T6) were similar to the control. By the 72-h sampling point, only the treatment with PZQ administered over three consecutive doses (T4) differed from the control ($P < 0.05$).

3.3. Neobenedenia girellae life cycle monitoring (Experiment 2)

The first signs of parasitism in experiment 2 by *N. girellae* in *S. dumerili* juveniles were observed on July 3, 2023, as water temperatures rose above 22 °C. Severe parasitism, leading to symptoms such as exophthalmia, haemorrhages, keratitis and secondary infections, developed in the untreated tanks between biweekly freshwater baths.

N. girellae adults collected from the fish spawned within 24 h at a temperature between 25 and 27 °C, with a mean fecundity of 58.82 ± 33.59 eggs/day/adult. Adults survived up to 72 h without a host, and egg hatching occurred within 4 days in the 300 mL beakers (26.5 ± 2.4 °C) and within 8 days in the plates at an average temperature of 24.8 ± 2.86 °C (Fig. 3). Oncomiracidia died within 48 h without a host.

The average egg area and perimeter ($n = 30$) were 0.01 ± 0.00 mm^2 and 0.44 ± 0.04 mm, respectively, while the average total length of adults ($n = 10$) was 3.96 ± 0.68 mm.

3.4. Incidence of N.girellae in relation to treatments (Experiment 2)

The different treatments significantly impacted the levels of *N.girellae* parasitism in the fish (Fig. 4), demonstrating consistency among replicates. While all PZQ protocols reduced the incidence of *N.girellae* adults, the treatments with divided doses (T3 and T4) were the most effective, with the incidence of *N.girellae* in T4 (PZQ 0.75 %-3) treated fish reduced to nearly zero (99.8 % efficacy, Table 5).

This was also reflected in the number of eggs counted daily on the polyester threads placed in the tanks (Fig. 5).

4. Discussion

4.1. Experiment 1

Concerning growth and feed utilization, the reduced performance in treatments 1 to 4, with no statistical differences, could have been caused by the limited tank volume (300*L*) relative to the fish weight range (130–216 g, with an average of 168 ± 20 g), even though rearing density was relatively low (<7 Kg/m^3). In fact, fish in the T5 (PZQ 0.75 %-2) and T6 (PZQ 0.75 %-1) treatments, with a weight range of 85–141 g (average of 110 ± 15 g), exhibited almost double SGR and WG compared to larger fish.

Therefore, this study, which aimed to provide initial insights into the kinetic profile of PZQ as a potential treatment to control *N. girellae* parasitism in *S. dumerili* juveniles within the EU, found that the PZQ protocols did not adversely impact fish growth or feed conversion rates. However, to further optimize growth during experimental trials, it is recommended to use larger tanks (at least 500 L) for juveniles exceeding 100 g in weight. Regarding PZQ levels in muscle, in treatments where PZQ was administered over 2–3 days (T3, T4, and T5), fish received a dose just 24 h before sampling, which explains their higher PZQ muscle concentrations at the 48-h sampling point. However, for treatments with 48 effective hours since the last administration (T2 and T6), PZQ levels in muscle were comparable to the control group, regardless of the dose (450 mg/kg in T2 vs. 150 mg/kg in T6).

At the 72-h sampling point, only treatment T4 (PZQ 0.75 %-3) showed PZQ levels statistically different from the control, as it was the only group to receive a dose the day before sampling. These results

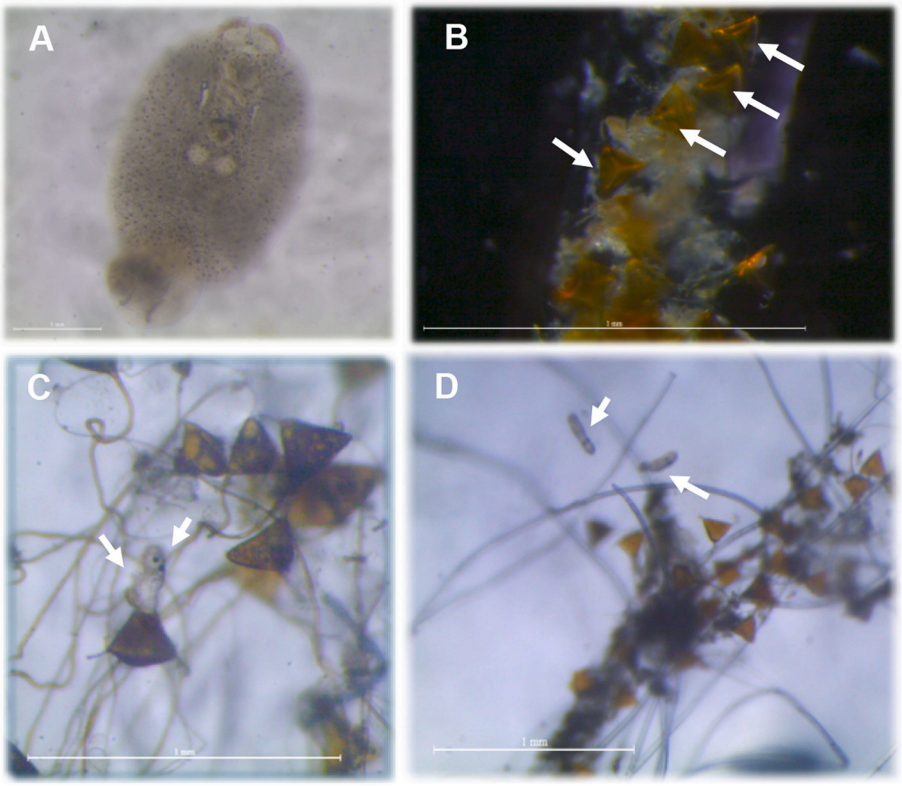

Fig. 3. Stages of *N.girellae* live cycle. A) Adults of *N. girellae*; B) Eggs (white arrows) on polyester threads; C) Hatching (white arrows); D) Free oncomiracidia (white arrows). Bars = 1 mm.

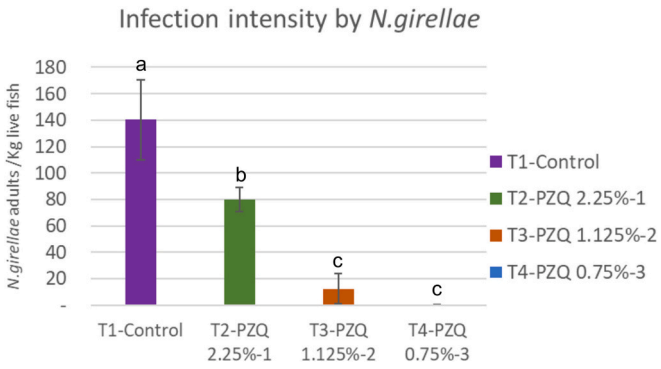

Fig. 4. Number of *N.girellae* adults per Kg of live fish, detached after freshwater baths at the end of the experiment, according to treatment (Experiment 2, n = 10 microchipped fish per treatment divided in two tanks). Columns with different superscripts indicate significant differences ($P < 0.05$). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel; T1-Control; control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

confirm that PZQ has a short persistence in *S. dumerili* muscle, with concentrations primarily influenced by the time since the last administration rather than the cumulative dose.

Kogiannou et al. (2021), also reported similar PZQ levels in muscle 72 h post-treatment, with levels falling below the limit of quantification by the fourth day in *S.dumerili* juveniles reared at 24 °C and fed 60 mg/Kg live fish over 5 days (cumulative dose of 300 mg of PZQ).

Table 5
Efficacy (%) of PZQ protocols against *N.girellae* in the *S.dumerili* juveniles (Experiment 2, n = 10 microchipped fish per treatment divided in two tanks).

	T1-Control	T2-PZQ 2.25 %-1	T3-PZQ 1.125 %-2	T4-PZQ 0.75 %-3
Efficacy (%)	–	42.86 ± 6.45^{a}	91.23 ± 8.17^{b}	99.80 ± 0.28^{b}

Columns with different superscripts indicate significant differences ($P < 0.05$). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel; T1-Control, control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

Short muscle withdrawal periods for oral PZQ at similar doses have also been reported in species like gilthead seabream (*Sparus aurata*) (Kogiannou and Rigos, 2021) and rockfish (*Sebastes schlegeli*)) (Kim et al., 2003), where PZQ was undetectable at 48 h post-treatment.

Regarding muscle concentrations at the different sampling points, the results of this study align with those reported by Kogiannou et al. (2021) in *S.dumerili* juveniles reared at 24 °C and fed 60 mg/Kg live fish for 5 days. However, compared to gilthead seabream reared at 25 °C and fed 150 mg PZQ/Kg live fish for 3 consecutive days (Kogiannou and Rigos, 2021), PZQ muscle concentrations in *S.dumerili* juveniles were higher at 24 h after treatment (~379 ng/g in this study versus 40 ng/g in seabream). Additionally, compared to rockfish reared at 20–21 °C and fed 400 mg PZQ/Kg live fish in a single dose (Kim et al., 2001), the PZQ concentrations in *S.dumerili* juveniles fed a comparable dose (T2-PZQ

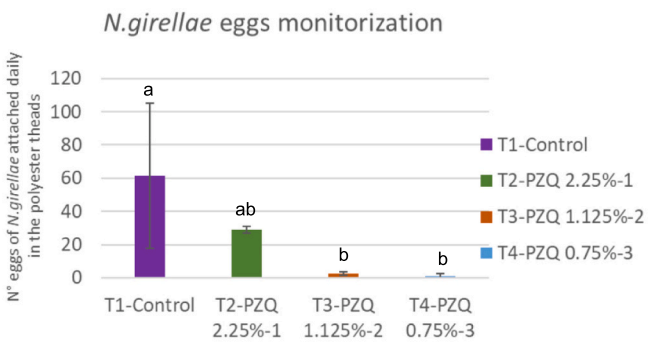

Fig. 5. Average number of *N.girellae* eggs counted daily in the polyester threads placed in the experimental tanks during the experimental period (Experiment 2, n = 10 microchipped fish per treatment divided in two tanks). Columns with different superscripts indicate significant differences ($P < 0.05$). Data expressed as means ± standard deviation. Abbreviations: PZQ, praziquantel; T1-Control, control treatment; T2-PZQ 2.25 %-1; treatment offering commercial diet +450 mg PZQ/kg live weight in a single dose; T3-PZQ 1.125 %-2, treatment offering commercial diet +225 mg PZQ/kg live weight 2 consecutive days; T4-PZQ 0.75 %-3, treatment offering commercial diet +150 mg PZQ/kg live weight 3 consecutive days; T5-PZQ 0.75 %-2, treatment offering commercial diet +150 mg PZQ/kg live weight 2 consecutive days; T6-PZQ 0.75 %-1, treatment offering commercial diet +150 mg PZQ/kg live weight in a single dose.

2.25 %-1) were lower (~329 ng/g vs 3610 ng/g). This variability in PZQ accumulation is consistent with previous reports of differences based on host species and environmental conditions like temperature (Bader et al., 2019).

It is important to note that comparisons have been made based on theoretical dosages, as the PZQ levels in the diets were not determined in the referenced studies to verify actual concentrations. This highlights the importance of determining PZQ levels in experimental diets to accurately assess the drug's kinetic profile and accumulation patterns.

Oil-coated drug administration is a common practise in aquaculture for medicated dry diets (Ranjan et al., 2017). However, in this study, there was a reduction of around 50 % between the theoretical and measured doses of PZQ, likely due to losses during feed handling and low adsorption of the oil-coating in high-fat feeds. Furthermore, drug leaching into the water can be significant, depending on factors such as the chemical's water solubility, the time feed remains in water before consumption, and the pellets' size (Ranjan et al., 2017). Therefore, more studies are needed to determine leaching patterns of oil-coated PZQ diets and to improve coating protocols to ensure accurate dosing and minimise environmental impact.

4.2. Experiment 2

The record temperatures in the North Atlantic in 2023, which were 2 degrees higher than expected (Climate Change Institute, 2023), worsened *N.girellae*, infestations, leading to severe outbreaks within just two weeks between freshwater baths in untreated control groups. Consequently, oral treatment intervals were adjusted to weekly in Experiment 2 (compared to fortnightly in Experiment 1) based on water temperature.

Regarding *N.girellae* life cycle monitoring, the survival without a host (both for adults and oncomiracidia) and egg hatching periods were similar to those reported in previous studies (Hirayama et al., 2009; Fernández Montero, 2020). However, fecundity and adult size in this study were lower than in other reports (Hirazawa et al., 2013; Fernández Montero, 2020). This may have been due to the more frequent freshwater baths in this study, which likely limited adult longevity and consequently, their size and fecundity (14 days between treatments here vs 21 and 30 days in the referenced studies).

As for the effects of experimental protocols on *N.girellae* incidence in *S.dumerili* juveniles, clear differences were observed between treatments. The protocols that split the PZQ dose over multiple days (T3-PZQ 1.125 %-2 and T4-PZQ 0.75 %-3) demonstrated the highest efficacy. The nearly 100 % efficacy of the longest protocol (T4-PZQ 0.75 %-3) aligns with reports of >80 % efficacy in chub mackerel (*Scomber japonicus*) treated with a similar protocol (150 mg PZQ/Kg for 3 days) (Yamamoto et al., 2011). This may be due to the short persistence of PZQ in fish tissues, as discussed earlier, and the *N.girellae* ability to detach and survive in the environment before reattaching to a new host (Bader et al., 2019).

While PZQ efficacy against monopisthocotylean eggs is unclear (Morales-Serna et al., 2018), the control of adults achieved through the studied PZQ protocols resulted in a significant reduction in the number of eggs counted on the polyester threads, helping to disrupt the *N.girellae* life cycle under the conditions of this experiment.

5. Conclusions

The oral praziquantel (PZQ) protocols were well tolerated by *S. dumerili* juveniles, with no adverse effects on growth or feed acceptance. PZQ levels in muscle returned to baseline within 48 h, and the high efficacy (99.8 %) of the T4-PZQ 0.75 %-3 protocol highlights that dividing the dose over three consecutive days is the most effective strategy for controlling *N. girellae* infestations.

The short withdrawal period further supports PZQ as a safe and practical treatment option for use in EU aquaculture. Future research should prioritize refining coating protocols to ensure accurate dosing and reduce environmental impact, as well as focus on long-term fish health monitoring and the scalability of these protocols for large-scale application.

CRediT authorship contribution statement

Raquel Quirós-Pozo: Writing – original draft, Investigation, Formal analysis, Data curation. **Christian Monzón:** Investigation. **Sarah Montesdeoca-Esponda:** Writing – review & editing, Investigation, Formal analysis, Data curation. **María Esther Torres-Padrón:** Investigation, Formal analysis, Data curation. **Javier Roo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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