Institute of Parasitology, Biology Centre CAS
Folia Parasitologica 2025, 72: 013
doi: 10.14411/fp.2025.013

Folia Parasitologica
http://folia.paru.cas.cz

Research Article OPEN ACCESS

Fecundity of the zoonotic nematode *Anisakis pegreffii* cultivated *in vitro*

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Abstract: The zoonotic marine nematodes of the genus *Anisakis* Dujardin, 1845 are the causative agents of anisakiasis, a parasitosis that has been increasingly reported in Europe over the past decade due to the more frequent consumption of lightly processed or raw seafood. While the life cycle in the marine environment is relatively well-known, an *in vitro* life cycle has recently been established with the goal to serve as a model for a better understanding of the functional biology of the nematode and consequent devising of strategies for its detection and inactivation. However, the reproductive capacity of the nematode has not been investigated so far, although it is an important parameter for epidemiological modelling or risk assessment studies. To measure the fecundity of *Anisakis pegreffii* Campana-Rouget et Biocca, 1955, type I larvae were obtained from naturally infected blue whiting *Micromesistius poutassou* (Risso) from the Adriatic Sea (Croatia) and cultured to the adult stage in Schneider's insect *Drosophila* medium supplemented with 10% chicken serum (n = 30 in triplicate). Larvae reached stage 4 (L4) by day 4 post-incubation (dpi), followed by molting to the stage 5 (L5) after 15 days and transition to the adult stage, characterised by production and expulsion of eggs on day 17 dpi. The fecundity of the adults was quantified by the daily number of eggs expelled per female, as well as their hatchability. Eggs were detected from 17 to 133 dpi but started hatching only from 44 dpi. Over the next 51 days, the eggs typically hatched into L2 larvae within 5–7 days. Average fecundity peaked at 100 dpi with 44,125 eggs/day/female and a sex ratio of 1 : 2 to 1 : 3. Cumulative mortality of cultured animals reached 60, 50 and 53% for the triplicates at 133 dpi, whereupon the experiment was terminated as only unfertilised eggs were produced.

Keywords: anisakiasis, *Anisakis* spp., *in vitro* life cycle, reproductive capacity, risk assessment

Due to an increase in cases of anisakiasis and anisakidosis in Europe, two clinical diseases in humans caused by species of the genus *Anisakis* Dujardin, 1845 and those of other genera of the family Anisakidae Raillet et Henry, 1912, respectively, more research efforts have been invested in the study of marine nematodes of the family Anisakidae (Bouwknegt et al. 2018).

The former disease usually results from the ingestion of viable third-stage larvae (L3) of *Anisakis simplex* (Rudolphi, 1809) *sensu stricto* (s.s.) and *Anisakis pegreffii* Campana-Rouget et Biocca, 1955, the latter also from the ingestion of larvae of *Phocanema decipiens* (Krabbe, 1878) *sensu lato* (s.l.) and *Contracaecum osculatum* (Rudolphi, 1802) s.l. In both cases, infection is facilitated by the consumption of contaminated raw or undercooked fish and cephalopods, which serve as intermediate hosts.

It has been established that over 20,000 people worldwide are affected by anisakidosis. For every 100,000 individuals, approximately 0.3 cases of anisakidosis are recorded (Bao et al. 2019). Between 1965 and 2022, 762 cases were reported, affecting people aged 7 months to adults over 80 years old. Anisakidosis has been recorded in 34 countries, with Spain, South Korea and Japan having the highest number of documented human cases (Cha and Ock 2012, Shamsi and Barton 2023). Although around 500 cases are reported annually in Asia, particularly in Japan, it is estimated that the actual number may exceed 20,000 cases due to under- or misdiagnosis (Sugiyama et al. 2022).

In Europe, around 500 cases are diagnosed each year, with Spain accounting for more than 150 of these cases. However, estimates suggest much higher numbers: up to 8,000 cases annually in Spain from the consump-

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tion of anchovies alone and possibly between 10,000 and 20,000 cases based on hospital data (Bao et al. 2019, Domingo-Hernández et al. 2023). These gaps highlight the widespread underdiagnosis and emphasise the urgent need for greater awareness and improved diagnostic methods at a global level.

Recent advances in the establishment of an *in vitro* life cycle have opened avenues for a more comprehensive study of the biological and physiological traits of *A. pegreffii* and *A. simplex* (Mladineo et al. 2023a, Moratal et al. 2023). In general, these advances are promising for screening potential drug targets and performing pathogenicity and virulence tests. In addition, insights from the *in vitro* life cycle can contribute to the understanding of developmental timelines, environmental factors influencing parasite survival and transmission, and the modelling of transmission dynamics. The integration of specific parameters from the *in vitro* life cycle enables the development of epidemiological models and risk assessments that increase the accuracy in predicting disease prevalence and contribute to the development of targeted control strategies.

However, there are still many reproductive features in the life cycle of *Anisakis* spp. that need to be clarified. For example, there are species-specific differences in fecundity between *A. simplex* (s.s.) and *A. pegreffii*, which produce an average of 29,914 (± 27,629) and 24,371 (± 12,565) eggs per day from a single female, respectively. Nevertheless, egg fertilisation appears to occur later than the onset of egg ejection in both species (Moratal et al. 2023), suggesting that maturation of females and males does not occur simultaneously, with female maturation slightly ahead of that of males.

The aim of this study was to validate the previously described *in vitro Anisakis* assay with a larger number of animals and to focus on certain reproductive characteristics of the parasite, such as the life span of *A. pegreffii*, its fecundity (number of eggs expelled per day) and the onset and cessation of egg hatchability. Improving knowledge of the reproductive characteristics of this zoonotic nematode, including reproductive characteristics and lifespan, will help to design and enhance future epidemiological modelling and risk assessments.

MATERIALS AND METHODS

Sample collection

Third-stage (L3) larvae of *Anisakis* spp. type I were collected from the visceral cavity of naturally infected blue whiting (*Micromesistius poutassou* [Risso]) caught in the Croatian Adriatic Sea and shipped by express courier in filtered and autoclaved seawater to the Institute of Parasitology in the Czech Republic. Upon arrival at the laboratory, larvae were checked for viability under a stereomicroscope and incubated three consecutive times in M9 buffer containing 2% penicillin/streptomycin (PS) (Sigma, Aldrich, Missouri, USA, P4333–20 ml) and amphotericin B (Sigma, A2942–20 ml) for 30 minutes in the dark at room temperature for decontamination. A subsample of 30 L3 was identified as *Anisakis pegreffii* by genotyping the ITS as previously described (Moratal et al. 2023).

In vitro culture

A total of 30 infective third-stage larvae (L3) were placed in Schneider's insect *Drosophila* medium supplemented with 10% chicken serum and 2% antibiotics in three biological replicates (200 ml autoclaved glass bottles B1, B2 and B3) according to a previously published protocol to reach the adult stage (Mladineo et al. 2023a). The bottles were kept at 37 °C in an atmosphere of 5% CO_2 and the transition from the isolated L3 stage to the subsequent stages was monitored every two days under a stereomicroscope (Zeiss, Stemi 305, Oberkochen, Germany), where changes such as size increase, colour change, development of the intestine and labial structures, loss of mucron and the ensheathment, and shedding of the cuticle were assessed and noted. The medium was aseptically replaced every two days and dead larvae or adults were removed at the same time.

Egg detection and quantification

The media were examined regularly to determine the onset of expulsion of eggs starting from the 14th day post incubation (dpi) of L3. The media were collected from the bottles into 50 ml tubes and centrifuged at 22 *g* for 20 minutes at 19 °C. After discarding the supernatant, 2 µl of the pellet were placed on a microscope slide and examined under an inverted light microscope (Olympus, CKX53, Tokyo, Japan) to detect the presence of eggs. For the quantification of eggs, the medium was similarly collected in 50 ml tubes and centrifuged under the same conditions.

Once the supernatant was removed, the pellet was washed twice with 5 ml of autoclaved sea salt solution (SSS), prepared by dissolving 31.73 g of Sea salts NutriSelect Basic (Sigma, S9883–500 G) in 1000 ml of distilled water. After centrifugation, the eggs were resuspended in autoclaved SSS and passed through a 70 µm-pore nylon cell strainer (Corning, 734–2761).

After a final centrifugation, the supernatant was discarded and the eggs were resuspended in 1–3 ml SSS, adjusting the volume according to the number of eggs and using more SSS for larger egg pellets. The resuspension was thoroughly mixed by pipetting and then transferred to a Bürker chamber. Quantification of the eggs was carried out under a light microscope three times a week, following the standard protocol for counting blood cells in the Bürker chamber as described by Moratal et al. (2023).

Eggs/ml = (total eggs counted × 10,000 eggs/ml)/number of squares counted

Total eggs/medium = volume of sample containing the eggs × total eggs/ml

Fecundity

Fecundity, measured as the number of eggs produced by each female within a day, was calculated separately for each biological replicate. To verify the reproductive characteristics of adult *A. pegreffii*, the number, sex and date of adult specimens removed from the medium due to mortality were recorded in addition to the egg count. These data allowed correlation of egg production with the sex ratio within each replicate. The sex ratio was calculated by dividing the number of females and males and expressing the results as a ratio. The daily egg count per reproductive female was plotted based on the total number of adults initially placed in culture and the calculated sex ratio at each count.

Egg hatching

To evaluate the duration of fertility of the females and the hatchability of the eggs, the latter were collected and incubated in autoclaved sea salt solution at 19 °C in a 5% CO_2 atmosphere. The temperature of 19 °C was chosen as it reflects the average surface temperature of the Mediterranean Sea, which generally ranges between 18–21 °C, varying by region and season. This temperature is biologically relevant, given the widespread presence of *Anisakis* spp. in the Mediterranean and is consistent with conditions used in prior studies (Mladineo et al. 2023a), facilitating comparability with existing findings.

Egg collection for incubation and subsequent hatching began at 37 dpi, when the expelled eggs showed signs of embryonic development under the microscope (Mladineo et al. 2023a). After eggs were collected for quantification, adults were placed in 50 ml of fresh medium for two hours to allow expulsion of new eggs that would not be affected and potentially damaged by the acidic culture conditions.

This new medium was drained from the tube containing the adult *Anisakis* into a 50 ml cylindrical tube and centrifuged at 22 *g* for 20 minutes at 19 °C. The supernatant was discarded and washed twice in 5 ml SSS under the same conditions. Subsequently, 2 ml SSS was added to the pellet and the solution was filtered using a sterile 70 µm Biofil Cell Strainer. The eggs were collected in a six-well plate, additional SSS was added to reach a total volume of 3 ml, and the plates were incubated at 19 °C and checked daily for hatching.

Statistical analysis

Statistical differences between the three replicates in terms of fecundity and cumulative mortality were analysed using a one-way ANOVA test, and group differences were evaluated using Tukey's HSD *post-hoc* pairwise comparisons.

RESULTS AND DISCUSSION

***In vitro* culture and development of larvae**

The development of the larvae of *Anisakis pegreffii* followed a well-established timeline. At 4 dpi, the larvae begin to transition to the fourth stage (L4), which is characterised by significant morphological changes, such as an increase in length, a change in the appearance of the intestine, which becomes coiled, a change in body colour and the shedding of the previous larval cuticle. At 15 dpi, the larvae shed the cuticle again, indicating their moult into the fifth (L5) or preadult stage. After 2 days (17 dpi), the onset of the adult stage was evidenced by the first expulsion of eggs. This marks the transition of the larvae to reproductive maturity, becoming capable of producing and releasing viable eggs into the environment. In contrast to the findings of Audicana et al. (2003), the L4 larvae of *Anisakis simplex*, and particularly *Phocanema decipiens*, take approximately 30 days to reach reproductive maturity after transitioning from the L3 stage. In most cases, the larvae complete development into the adult stage, with only rare instances of incomplete development. Furthermore, the first detection of eggs, indicating reproductive maturation of *A. simplex* s.l., was observed approximately 24 dpi in the research by Moratal et al. (2023), when cultured *in vitro* under conditions similar to those in this study.

The ability of *A. pegreffii* to reach reproductive maturity within 17 days, together with the detection of the first eggs at 21 dpi in the study by Moratal et al. (2023), contrasts with the 24 dpi and 30 dpi observations for closely related species in the previous studies. This highlights the rapid life cycle of this parasite, having significant implications for its transmission dynamics.

At 36 dpi, the majority of the cultured *A. pegreffii* had reached adulthood, but there was asynchrony in the development of some larvae that had remained in the L4 stage. This variability in developmental progression within the population could be influenced by several factors, including genetic background, possible mechanical damage during isolation and the long prepatent period in the paratenic host. Of the 90 L3 larvae initially cultured, a total of 5.6% L4 larvae were recovered from the media as they did not reach adulthood.

The presence of both rapidly and slowly developing specimens within the same cohort suggests a degree of developmental plasticity, which may indicate that non-maturing larvae do not infect the final host but are likely to be excreted into the environment via faeces.

This is consistent with a study by Bušelić et al. (2019), in which rats were infected with *A. pegreffii* L3. Six hours after infection, 80 and 90% of the L3 larvae were expelled from the host in the pre-infection and experimental infection, respectively.

Furthermore, not all larvae, although belonging to the same cohort, caused infection or tissues damage in the host within 24 hours of infection, with results varying depending on the parasite's migration route in the host.

Understanding the asynchrony of larval growth in relation to infection success could be important for mapping virulence factors or lack thereof.

Eggs expulsion and quantification

The first eggs were detected in the medium from day 17 dpi and continued to be observed until day 133 dpi, indicating 3.8 months of reproductive activity. However, the fertilised eggs that successfully hatched were not produced until 27 days after the first eggs were detected in the medium. Over the following 51 days, from 44 to 95 dpi, the eggs continued to be fertilised and hatched into second-stage larvae (L2).

In contrast to a study by Ferris et al. (1996), the marine nematode *Chromadorina persegnis*, when cultured in nematode growth media (NGM) at 20 ± 1 °C, produced eggs from 10.6 dpi, while the terrestrial nematode *Caenorhabditis elegans* was able to produce viable eggs from 5.5 dpi. Furthermore, in the same study, species of the family Cephalobidae, including *Acrobeloides bodenheimeri*, *A. buetschlii* and *Cephalobus persegnis,* and species of the family Rhabditidae (*Bursilla labiata*, *C. elegans*, *Cruznema tripartitum* and *Rhabditis cucumeris*) exhibited egg production times of 10.6 to 21.4 dpi and 5.5 to 9.9 dpi, respectively.

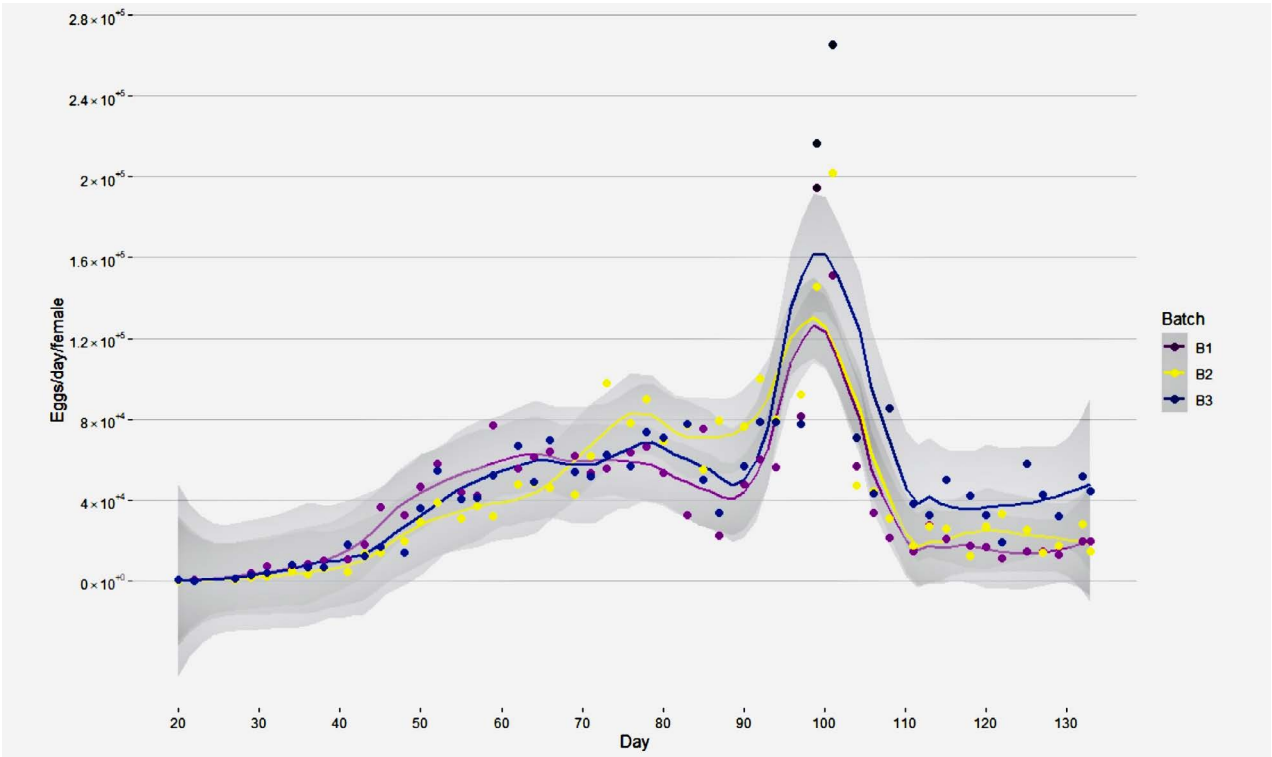

Fig. 1. Fecundity of *Anisakis pegreffii* Campana-Rouget et Biocca, 1955 (eggs/day/female) in *in vitro* culture calculated for each biological replicate (B1, B2, B3). Each replicate's smoothed trends are represented by the coloured lines (purple, yellow and blue), while the corresponding points display daily observations. The 95% confidence interval is represented by light grey shading while the 50% confidence interval is represented by dark grey shading. Fecundity peaks around day 100, after which egg production starts to decline.

The females of *A. pegreffii* produced eggs for a further 38 days, but these were not fertilised despite the presence of the males, suggesting a change in the gamete material or a possible deterioration in the viability of the males' sperm. This is consistent with previous observations that a change in sperm number, motility, a decrease in sperm viability and insufficient sperm transfer could lead to a decrease in egg fertilisation rate, even under optimal environmental or culture conditions (Bird and Bird 1991, Cutter 2004).

Typically, the hatching process in *A. pegreffii* takes 5–7 days after incubation of the eggs, which is a fixed characteristic for the development of the eggs at the given temperature. Moratal et al. (2023) observed that the first egg expulsion for *A. pegreffii* starts at 21 dpi and lasts until 120 dpi (discontinued due to contamination). Viable fertilised eggs were present in the media from day 31 to day 86, with an estimated sex ratio of 3 : 2 (males : females). Despite slight differences in the sex ratio, no significant differences in the time of egg deposition were observed between this and the aforementioned study. The same nematode species was used in both studies and the larvae came from the same source, that mostly warrants a relatively homogeneous genetic background, while the culture conditions remained the same.

However, in this study, the fecundity of the worms peaked at 100 dpi (Fig. 1), with the following values observed for the three replicates: 194,775 (in B1), 202,038 (in B2) and 265,763 (in B3). The average fecundity during the experimental period was 39,884 (± 27,447) in B1, 42,602 (± 28,925) in B2 and 49,889 (± 33,848) in B3, measured as eggs per day per female. The mean total fecundity was 44,125 eggs per day per female. No statistically significant difference in fecundity was observed between replicates ($p = 0.34$), indicating that the experimental conditions were consistent. Furthermore, fecundity observed by Moratal et al. (2023) revealed an average production of 29,914 (± 27,629) and 24,371 (± 12,565) eggs/ day/ female in *A. simplex* and *A. pegreffii*, respectively.

These fecundity rates highlight the high reproductive potential of *A. pegreffii* which may contribute to its ability to effectively maintain and propagate its population. However, the fecundity data presented here differ from those in the study by Moratal et al. (2023), in which the average total fecundity of *A. pegreffii* reached 24,371 (± 12,565). Considering that 30 worms were cultured per flask using 50 ml medium in the present study compared to 57 worms using 80 ml medium, this difference in egg quantity could be due to the ratio of medium to worm. Lower worm density may have increased the availability of nutrients, possibly leading to more egg laying. This is consistent with the findings of Harvey et al. (2008), who suggest that the fecundity, timing of reproduction, lifespan and body size of nematodes are influenced by the availability of food and nutrients in their environment as well as population density.

The high fecundity of species of *Anisakis* can be contextualised by comparison with other nematode species. For example, Iglesias et al. (2002) reported that *Hysterothylacium aduncum* (Rudolphi, 1802) (Raphidascarididae), which infects marine and freshwater teleosts, has an average egg production of 27,015 eggs per female when

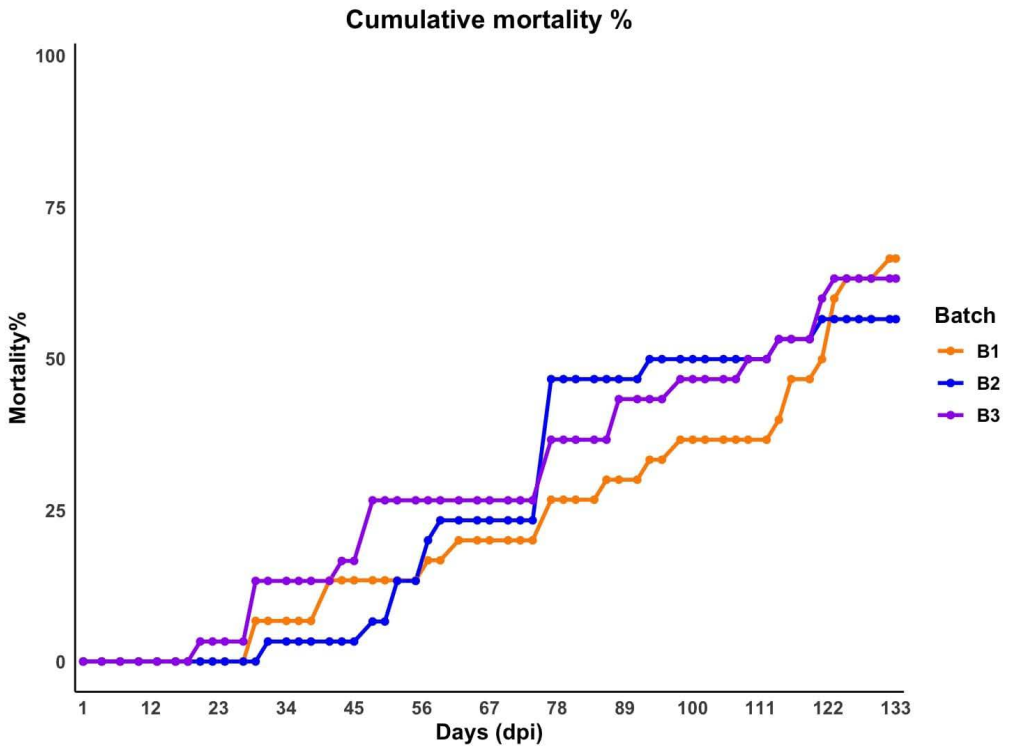

Fig. 2. Cumulative mortality (%) of three biological replicates (B1, B2, B3) of adults of *Anisakis pegreffii* Campana-Rouget et Biocca, 1955 cultivated in Schneider's insect *Drosophila* medium enriched with 10% chicken serum. Each replicate's trends are represented by the coloured lines (purple, yellow and blue), while the corresponding points display mortality observations.

cultured in RPMI growth medium at pH 4.0 and with 1% commercial pepsin.

In terrestrial environments, the female parasitic nematode *Trichinella spiralis* (Owen, 1835) (Trichinellidae), which infects pigs and is also responsible for trichinosis in humans, is capable of producing eggs that hatch internally and release approximately 1,500 larvae within their six-week life span (Crowley 2013). Each female of the parasitic *Haemonchus contortus* (Rudolphi, 1803) (Trichostrongylidae), which is prevalent in sheep and goats, can produce 10,000 eggs per day, from which a large number of free-living larvae from L1 to L3 can hatch (Herath et al. 2021).

Sex ratio and cumulative mortality

Each biological replicate consisted of 30 larvae with a similar sex ratio, affected only by a number of underdeveloped L4 that never reached reproductive maturity and were removed after death: B1 harboured 19 females, 9 males and 2 L4; B2 comprised 22 females, 7 males and 1 L4 larva, while B3 contained 19 females, 7 males and 4 L4 larvae. Consequently, a sex ratio of 1 : 2 (males: females) was calculated for B1 and 1 : 3 for B2 and B3.

The recorded cumulative mortality rates over time reached 60% in B1, 50% in B2 and 53% in B3, with no significant difference ($P > 0.05$) between the replicates (Fig. 2). In the previous study by Moratal et al. (2023), a cumulative mortality of 71% of adult worms was observed at the end of the experiment, which was probably due to fungal contamination by dead specimens. At the beginning of the experiment, the dead L4s removed from the medium were smaller compared to their conspecifics, suggesting that these worms were potentially underdeveloped and may have had a reduced fitness.

The first mortalities occurred 20 dpi and at 133 dpi almost half of the adults were still alive, indicating their longevity under artificial conditions with unlimited nutrient supply and no interaction with the host immune system or host-associated microbiota (Mladineo et al. 2023b). However, the experiment was interrupted at 133 dpi as eggs that were not fertilised were continuously produced. We hypothesise that this is unlikely in the natural environment, as the availability of nutrients could limit the survival of females that are unsuccessful at reproduction. However, it is also possible that with a more extensive pool of males in the final host, fertilisation would still be possible after 133 dpi.

Anisakis pegreffii exhibits adaptability to survive, develop and reproduce under *in vitro* conditions for a prolonged period of at least four months, with low variability between biological replicates. However, while *A. pegreffii* shows sustained viability in an insect medium over this long period, the time window for successful fertilisation of eggs and subsequent hatching of second-stage larvae (L2) is restricted to a limited period (e.g., 44–95 dpi). Beyond this time frame, the viability of the eggs for hatching decreases, which limits the feasibility of experiments that rely on viable larvae.

Despite this time constraint, the observed cumulative mortality among adult worms ensures a satisfactory yield of fertilised eggs suitable for robust downstream experiments. This highlights the importance of careful experimental design and replication to compensate for natural mortality rates

when maintaining *A. pegreffii* populations in the laboratory. By taking these mortality rates into account and ensuring a sufficient supply of adult worms in the study design, one can reliably obtain the required number of fertilised eggs needed for further applications.

Acknowledgements. The internship of H.N.B was supported by the Erasmus Mundus Joint Masters Scholarship. Futhermore, this research was supported by national funds through GACR, grant No. GA23-07990S, Secretomics: exploration of intercellular communicators between parasites and their host, to Roman Kuchta.

Author contribution. H.N.B. performed the experiment, data collection and analysis, visualization, and writing of the manuscript. S.M.M. and J.H. contributed by reviewing and editing the manuscript. I.M. conceptualized the study, provided resources, supervised the experiment, data analysis, and visualization, including writing of the manuscript.

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Received 22 November 2024 Accepted 26 March 2025 Published online 14 May 2025

Cite this article as: Birikorang H.N., Martínez S.M., Hrabar J., Mladineo I. 2025: Fecundity of the zoonotic nematode *Anisakis pegreffii* cultivated *in vitro*. Folia Parasitol. 72: 013.