Journal of
Marine Science and Engineering

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Behavioural Changes of *Anisakis simplex* (s.s) Third-Stage Larvae Induced by Biotic and Abiotic Factors in the Fish and Mammalian Hosts: In Vitro Studies

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Abstract: The marine parasitic nematode *Anisakis simplex* has a complex life cycle including marine mammals (mainly cetaceans) as definitive hosts, invertebrates (e.g., copepods and euphausiids) as the first paratenic hosts, and a wide range of fish species acting as second paratenic hosts. During the life cycle, the larva encounters a wide range of biotic (host immune factors and microelectric activity) and abiotic conditions (temperature and pH). We hypothesized that these factors may explain the differential behaviour of the nematode, recognized during the different life cycle stages. In this study, third-stage larvae (L3) of *A. simplex* were isolated from freshly caught Atlantic herring (*Clupea harengus*) from FAO zone 27. We exposed nematodes to different pH values (pH 2 to 9) at different temperature levels (4, 14, 21 and 37 °C), electric currents (6 mA, 12 mA, 18 mA) and different concentrations of fish immune cells. The nematode larvae exhibited significantly differential behaviour (stretched non-aggregated, spiral non-aggregated and aggregated) and activity levels when exposed to the different physicochemical conditions. We recorded negative correlations between activity and pH (maximum at pH 2) and positive correlations between activity and temperature (maximum at 37 °C). The nematode larvae were affected when exposed to electricity and fish immune cells. Electric currents at 6 mA induced minor changes, but at 12 mA and 18 mA, the majority or all nematode larvae aggregated and rolled up into spirals. Exposure to leukocytes, isolated from rainbow trout head kidney and spleen, induced a similar concentration-dependent spiralling process in larvae. We discuss these behavioural patterns of *A. simplex* as adaptations to conditions encountered by the worm larvae during the different stages of their complex life cycle.

Keywords: nematode; life cycle; pH; temperature; electricity; immune cells

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Citation: Kumas, K.; Gonzalez, C.M.F.; Kania, P.W.; Buchmann, K. Behavioural Changes of *Anisakis simplex* (s.s) Third-Stage Larvae Induced by Biotic and Abiotic Factors in the Fish and Mammalian Hosts: In Vitro Studies. *J. Mar. Sci. Eng.* **2024**, *12*, 1546. https://doi.org/10.3390/jmse12091546

Academic Editors: Perla Tedesco and Marialetizia Palomba

Received: 30 July 2024
Revised: 27 August 2024
Accepted: 3 September 2024
Published: 4 September 2024

1. Introduction

The parasitic worm *Anisakis simplex* (Phylum Nematoda, Superfamily Ascaridoidea, Family Anisakidae, genus Anisakis) is also known as herring worm or whale worm due to its complex life cycle involving both fish and cetaceans [1–3]. The parasite is zoonotic and may infect humans ingesting raw or semi-raw fish containing live third-stage larvae. Adult worms inhabit the stomach of a marine mammal (the definitive host) from where they release eggs, which leave with host feces to the marine environment. Eggs embryonate, and when hatching, third-stage larvae are released and thereby exposed to ingestion by copepods and euphausiids, in which the larvae infect the hemocoel [1,4]. The infected crustacean serves as the first paratenic host, but when a fish (second paratenic host) ingests this, the larva passes from the gastrointestinal tract to the body cavity and become encapsulated in its mesenteries along the intestine, pyloric caeca, stomach, liver, gonads, and even musculature [5]. The paratenic host may then be preyed upon by a marine mammal, whereafter L3 larvae will moult twice and obtain the adult stage in its stomach of these definitive hosts [6]. During these life cycle stages, *A. simplex* faces different physicochemical conditions. Thus, in the marine mammal stomach, nematodes encounter a temperature of more than 35.5 °C [7] and pH 2 [8]. When penetrating the paratenic fish host, nematode larvae become exposed to pH 6–7 in the fish organs [9] as well as in the fish flesh [10]. During its free-living larval stage, the nematode encounters seawater at pH 8–9 [11] and a similar pH in the copepod host [12]. Electrophysiological studies in fish have documented that electric currents can be measured in different body compartments, which indicate that larvae in fish will be exposed to different electric currents during their migration in the fish [13,14]. Thus, in the present study, we elucidate the reaction of larvae exposed to current levels found in fish. Further, when entering the body cavity, the worm larva will be exposed to immune responses including host leukocyte colonization [15,16], and we have therefore investigated the behaviour of larvae exposed to different concentrations of cells from fish head kidney and spleen. In addition, dependent on the fish habitat, this parasite will be exposed to different temperatures because fish hosts are poikilothermic animals [17]. On a theoretical basis, we hypothesize that the parasite behaviour is adapted to different life cycle situations. To investigate this, the current study aims to describe *A. simplex* behaviour when exposed to different abiotic (temperature, pH, electricity) and biotic (fish immune cells) conditions.

2. Materials and Methods

2.1. Fish

To collect fish for parasite isolation, a total of 300 herring (total body weight of 115–332 g and total body length of 27–34 cm) were captured in the FAO 27 zone by commercial trawlers during the period spanning from February to June 2024. Immediately following landing, the fish were transferred to the University of Copenhagen under cooled conditions (on ice in a refrigerated lorry). In order to isolate leukocytes for the in vitro exposure of parasites to immune cells, we used disease-free rainbow trout (*Oncorhynchus mykiss*) (mean of total body weight of 7.6 g and body length of 8.9 cm). Fish were produced in a disease-free recirculated system based on hatching of disinfected certified eggs [18]. Prior to leukocyte isolation, the fish were transported to the university facility and acclimated in freshwater fish tanks at 16 °C for three weeks.

2.2. Parasitological Examination

Herring dissection was carried out for visual inspection according to Buchmann [19]. Nematode larvae were isolated from the body cavity and rinsed in water. Only larvae spontaneously leaving their encapsulation, exhibiting viability and high motility, were selected for the trials. Care was taken to avoid larval damage during manipulation, using forceps and spoons. A total of 1160 live and active nematodes were recovered and used in the analysis.

2.3. Morphological and Molecular Identification of Nematodes

Subsamples of the isolated nematode larvae were preserved in 96% ethanol (cat.no. 201160, KiiltoClean A/S, Assens, Denmark) and kept at 4 °C until processed for identification. Each worm was aseptically divided into three pieces (anterior, middle, and caudal). The anterior and caudal parts were mounted on microscope slides using mounting medium Aquatec (Merck, Brøndby, Denmark) for morphological identification. The middle part was used for molecular identification targeting rDNA and mtDNA by PCR and subsequent sequencing. Genomic DNA was purified by the means of a QIAamp DNA Mini Kit (cat.no. 61306, Qiagen, Hvidovre, Denmark) according to the manufacturer's instructions, except that 50 µL of elution buffer was used. PCR was performed in 60 µL volumes composed of 0.6 µL of DNA Polymerase, 6 µL of 10× Reaction buffer, 1.8 µL of 50 mM $MgCl_2$ (all three BIOTAQ DNA Polymerase, cat.no BIO-21060, Saveen & Werner ApS, Jyllinge, Denmark), 10 mM dNTP mix (Applied Biosystems™ GeneAmp™ dNTP Blend (100 mM)), (cat.no. 10085714, Fisher Scientific, Slangerup, Denmark), 6 µL of forward and reverse primers (Tag Copenhagen, Frederiksberg, Denmark) (both 10 mM) and finally 28.6 µL of RN'ase-free water (cat.no. 12060346, Fisher Scientific, Denmark). Primers used for the ITS region were PDG_18S_F5 (5′-CGATAACGAACGAGACTC-3′) according to [20] and reverse primer NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) according to [21]. Primers for the mt DNA were 211F (5′-TTTTCTAAGTTATATAGATTGRTTTYAT-3′) as the forward primer and 210R as the reverse primer (5′-CACCAACTCTTAAAATTATC-3′) according to [22]. PCR conditions for the ITS region consisted of one cycle of pre-denaturation at 95 °C for 5 min and 40 amplification cycles of denaturation at 95 °C for 30 s/annealing at 54 °C for 30 s/elongating at 72 °C for 1 min, followed by post-elongation at 72 °C for 7 min. With regard to the mitochondrial gene *cox2*, the condition was the same except for the amplification step, which was performed using a touch-down procedure using 2 cycles at 53 °C, 2 cycles at 51 °C, 2 cycles at 50 °C, 3 cycles at 49 °C, 3 cycles at 48 °C, 3 cycles at 47 °C, and 35 cycles at 46 °C, with each step for 45 s. The PCR products were visualized by 1.5% agarose gel electrophoresis. Products were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (cat.no. 28-9034-71, VWR International A/S, Søborg, Denmark), sequenced at Macrogen Europe, the Netherlands, and analyzed using the software CLC-Main Workbench v20.0.4 (QIAGEN, Hvidovre, Denmark).

2.4. Experimental Design

In order to study worm behaviour, the larvae were exposed to a series of abiotic and biotic factors including different pH levels, temperatures, electric currents, and concentrations of fish immune cells. During the experiment, nematode behaviour was assessed by visual observation.

2.4.1. Electric Stimulation at Different pH Levels

Electric stimulation of worm larvae was conducted by using 10 cm × 10 cm (5 mm thick) 1% agarose gels prepared with 10 mM UltraPure™ 1M Tris-HCI, pH 8.0 (cat.no. 1556802, Thermo Fisher Scientific, Roskilde, Denmark), and pH levels were adjusted from 2 to 9 with NaOH (cat.no. S8045, Merck, Denmark) and HCl (cat.no. 258148, Merck, Brøndby, Denmark). A well (diameter 25 mm) was punched out in the centre of the gel, in which larvae were placed. The agarose gel was chosen, as the material leads the current well and is inert without having an effect on the worms. The well ensured that worm larvae were not accidentally displaced during the study. Each exposure included 10 larvae in duplicate. Nematodes were exposed (5 min) to different electric current levels (0 mA [Control], 6 mA, 12 mA, 18 mA) in a multiSUBMaxi 200 × 100 and 200 × 200 mm electrophoresis chamber (cat.no. 9584670, Buch & Holm A/S, Herlev, Denmark), while nematode behaviour was observed. A buffer solution (with the same pH as the agarose gels) was added to cover the electrophoresis chamber.

2.4.2. Temperature at Different pH Levels

Larvae (10 for each exposure) were exposed in duplicate to different pH levels in Petri dishes (cat.no. AL-900315N, Dacos A/S, Esbjerg, Denmark) (diameter 12 cm) with 1% agarose gel with a central well (diameter 25 mm) in which larvae were placed. The gel and incubation fluid (10 mL in the well), as well as the agarose gels, were prepared from UltraPure™ 1M Tris-HCI, pH 8.0, adjusted with the use of NaOH and HCl from pH 2 to 9. Nematode behaviour was recorded at 0, 60, and 120 min, at different temperatures (4, 14, 21, 37 °C), in thermostat-regulated chambers.

2.4.3. Immune Cells

Head kidney leukocytes were isolated from rainbow trout, which were euthanized in an overdose of the anesthetic agent tricaine methane sulphonate MS222 (300 mg/L) (cat.no. A5040, Meck, Brøndby, Denmark). Thereafter, the head kidney (HK) and spleen (SP) were removed aseptically from the fish. The immune organs HK and SP were individually transferred to a 100 μm pore sized Falcon cell strainer (cat.no. 93100, Merck, Brøndby, Denmark) and leukocytes from individual organs were collected in a 100 mL glass beaker containing 600 μL of Leibovitz's L-15 Medium (cat.no. 11415049, Thermo Fisher Scientific, Roskilde, Denmark). Live cell counting of the cell solutions was performed microscopically (Leica Microsystems A/S, Brønshøj, Danmark) with a Neubauer hemocytometer (cat.no. BR717805, Merck, Brøndby, Denmark) based on a mixture of 10 μL of cell solution and 10 μL of Trypan blue (0.4%) (cat.no. 16520050, Thermo Fisher Scientific, Roskilde, Denmark). Nematode larvae were individually incubated to increasing concentrations of leukocytes from HK (5 larvae per concentration) and SP (5 larvae per concentration). The control groups were exposed to L-15 medium alone (without any cells). Nematode behaviour was recorded at 0, 10, and 30 min. All cell studies were performed at 19 °C.

2.5. Behavioural Reactions of Worm Larvae

Three different types of larval behaviour in the experimental groups were recorded as (1) stretched non-aggregated, (2) spiral non-aggregated, and (3) aggregated (Figure 1). The activity (motility) level of the worms was graded on a scale from 0 to 5: no motility (0), very low motility (1), low motility (2), medium motility (3), high motility (4), and very high motility (5). The behaviour and activity of the larvae were calculated as the percentage of all worms included in each experiment.

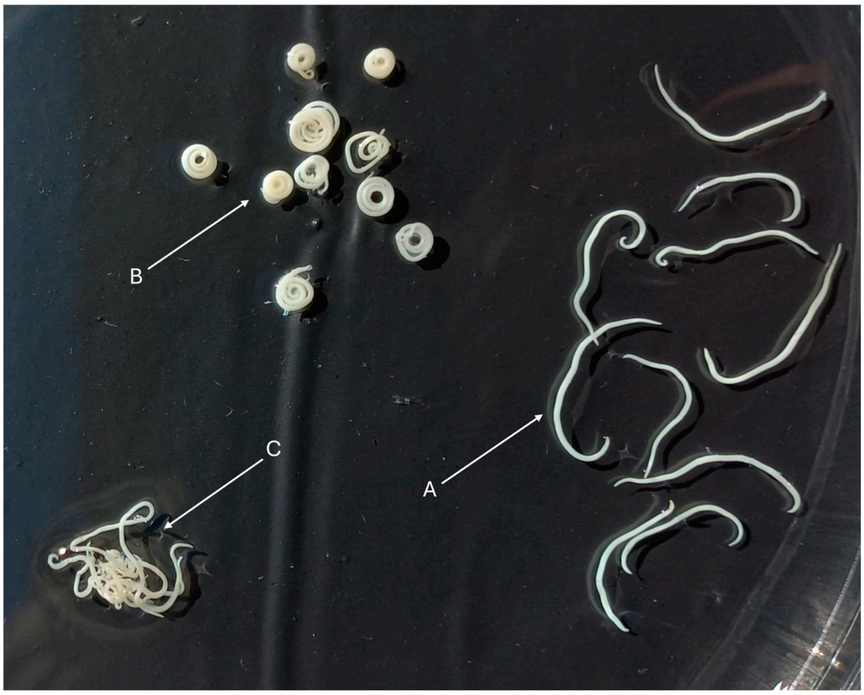

Figure 1. A: Stretched non-aggregated nematodes; B: spiral non-aggregated nematodes, C: aggregated nematodes.

2.6. Statistics and Calculations

Infection parameters were calculated according to [23]. Graph Pad Prism 10.2.3 (USA) was used for statistical analysis. All the statistical analyses were performed using the non-parametric Kruskal–Wallis with Dunn's multiple comparisons test, $p < 0.05$.

3. Results

3.1. Infection Levels of Herring Used for Worm Isolation

The nematodes were recovered from mesenteries in the body cavity of herring. They were located along organs such as the intestine, pyloric caeca, stomach, gonads, and liver. The prevalence of the infection was 100%.

3.2. Identification of Nematode Larvae

All nematode larvae were first identified at the *Anisakis* genus level by their morphology: larval tooth present, excretory pore anterior to nerve ring, ventriculus without appendage, no intestinal caecum, posterior end with mucron. They were then identified as *A. simplex* s.s. by molecular methods including ITS region (18S (partial)-ITS1-5.8S-ITS2-28S (partial)); 14 PCR products (GenBank accession numbers PQ108495 to PQ108508) were obtained, all 1411 bp long, including primer binding sites. Three of the products differed by having one heterozygote base at bp 632 (G→Y) and one at bp no. 765 (T→Y). These two variants exhibited 100% and 99.85 identities (excluding the primer binding sites) towards the GenBank acc.no. JX237370 (*A. simplex*) isolated from Denmark [16]. The 11 remaining ITS sequences were identical and showed 100% similarity. With regard to the mitochondrial gene *cox2*, we obtained 14 products (GenBank acc. nos. PQ126419 to PQ126432). They were all 630 bp long, including primer binding sites. Identities were more diverse, ranging from 98.80% to 100% (e.g., GenBank acc. nos. KT852498, GQ338428 and MW073763), excluding the primer binding site. They all encoded for identical amino acid sequences.

3.3. Behaviour Induced by Electric Stimulation at Different pH Levels

Worms with no electric stimulation at a specific pH served as control groups for each pH value. Control-group worm larvae showed a continuous medium motility and displayed a stretched-non aggregated behaviour. At 6 mA, significant changes in nematode behaviour were observed only at pH 8 and pH 9. When the current was increased to 12 mA, nematode larvae mainly aggregated or coiled up as a spiral (non-aggregated) except for a few stretched and non-aggregated specimens. At 18 mA, no stretched and non-aggregated larvae were present, as all the worms were either spiral non-aggregated or aggregated (Figure 2). Moreover, in the experiments, a decrease in activity levels was observed with the increasing current.

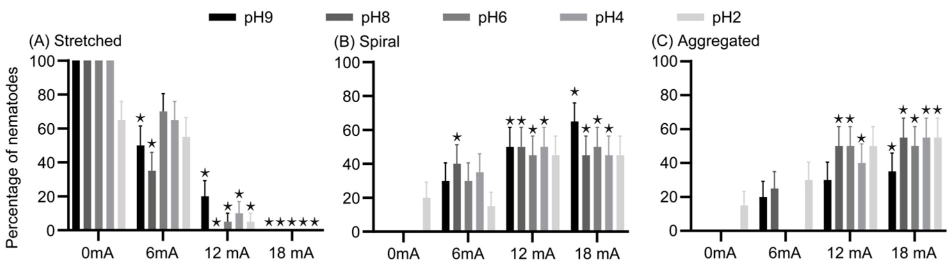

Figure 2. Nematode behaviour upon electric stimulation at different pH values. Asterisks above the bars indicate significant differences to the control (0 mA) at the specific pH values (Kruskal–Wallis with Dunn's multiple comparisons test, $p < 0.05$). Detailed data are presented in Supplementary Table S1.

3.4. Behaviour Induced by pH at Different Temperatures

The nematode activity was negatively correlated to pH values (maximum at pH2, minimum at pH9) and positively correlated with temperature (maximum at 37 °C) (Figure 3). The highest activity was observed among larvae at pH2 when kept at 37 °C, and the lowest activity was found at pH 8 and 9 at 4 °C.

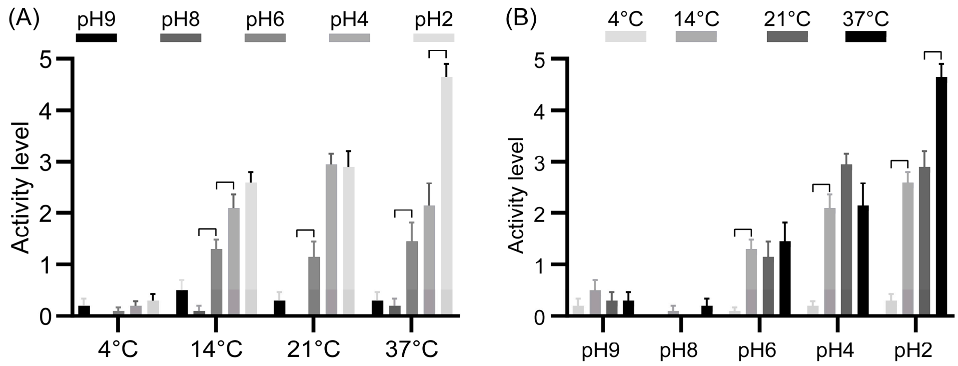

Figure 3. Activity levels of nematodes at different pH values kept at different temperatures (observation time point: 120 min). Panels A and B contain the same data but differently organized and with different comparisons. (**A**) Different pH values were compared at specified temperatures. (**B**) Different temperatures were compared at specified pH values. Brackets indicate significant differences between neighbouring groups (Kruskal–Wallis with Dunn's multiple comparisons test, $p < 0.05$). Detailed data for comparisons between all combinations of temperature and pH are presented in Supplementary Tables S2 and S3.

3.5. Behaviour Induced by Immune Cell Exposure of Nematode Larvae

Nematode larvae were incubated in different concentrations of rainbow trout leukocytes isolated from the head kidney and spleen of rainbow trout. In all groups, all nematodes were stretched and non-aggregated and motile at the start of the incubation period. The control larvae in L-15 medium without cells did not change their behaviour during the experiment. In contrast, after 10 min, some of the larvae exposed to cells changed their behaviour and coiled up into spirals. This process was even further expressed in all exposed larvae at 30 min. Behavioural changes occurred in cells from both the head kidney and spleen, although differences were noted. Thus, spleen cells induced a stronger coiling process compared to head kidney cells at a comparable concentration (1644 versus 1626 cells/µL (Figure 4)). Aggregated behaviour was not included as a possibility, because all nematodes were individually exposed (one worm per well). Activity levels decreased with time and with increasing leukocyte concentration.

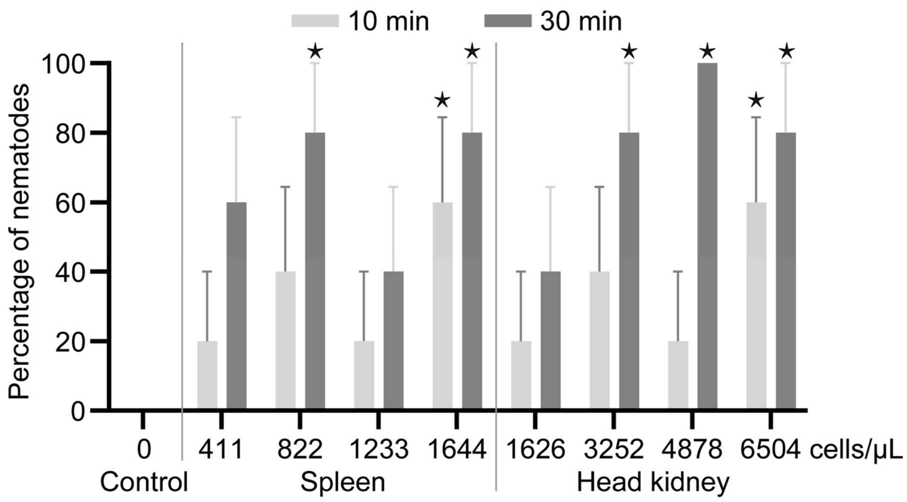

Figure 4. Nematode behaviour. Individual nematode larvae were exposed to immune cells (from spleen or head kidney) and behaviour (percentage of spiral nematodes) was recorded 0, 10, and 30 min after exposure. The time point 0 min had, in all cases, the value 0% and is not shown. *: Asterisks indicate significant differences to the control (0 cells/µL) (Kruskal–Wallis with Dunn's multiple comparisons test, $p < 0.05$). Comparisons between all combination of time points and numbers of immune cells are presented in Supplementary Table S4.

4. Discussion

The parasitic nematode *A. simplex* has a complex life cycle comprising the adult reproductive stage in the stomach of cetaceans, the egg and early larval stages in seawater, and larval stages in first and secondary paratenic hosts [1]. The environmental conditions encountered by the different life cycle stages vary considerably. The acidic and warm microhabitat in the homoiothermic mammal is highly different from the neutral and cold habitat in the poikilothermic paratenic hosts. The present study has elucidated how the parasite is adapted to these changing environments.

The behaviour of the parasite larva, residing as an inactive and encapsulated organism in the fish, changes significantly when exposed to a low pH and high temperature (37 °C) mimicking the conditions in the cetacean stomach. When a marine mammal ingests the fish host carrying infective third-stage larvae, the fish tissues will be digested in the digestive stomach solution consisting of hydrochloric acid (near pH 2) and pepsin at high body temperature (near 37 °C). However, the optimal reaction of worm larvae would be increased activity, moulting, and development to the reproductive stage. In the present study, the highest activity of the worm larvae was noted when pH decreased to 2, as in the stomach of the whale, and the temperature increased to 37 °C, which is near the body temperature of the marine mammalian. This can be interpreted as an adaptation to the reproductive period including mate finding and copulation in the whale stomach.

In the paratenic host, the larva must survive until the host is ingested by a definitive host, and this may be achieved by adopting coiling behaviour. Thus, the larvae are found aggregated in the mesenteries along the intestine, pyloric caeca, stomach, liver, gonads, and even musculature. It was noted that low electric currents, as may be found in the internal organs of fish [13,14], induced behavioural changes reflecting adaptation to a sedentary period in the fish. One possibility is that the electric current acts as a signal inducing the larva to attain a hypobiotic stage in the paratenic host awaiting ingestion by the definitive host. Another explanation is that coiling up into spirals in the fish host is a general defensive reaction when any noxious stimuli reach the sensory organs of the parasite. A third possibility is that larvae navigate according to electric signals in the host. Thus, the larval migration in the fish host following infection may be directed (at least partly) by the different electric voltages and currents known to exist in fish organs.

The coiling process of the nematode larvae was an even more pronounced behavioural reaction when the larvae were exposed to host immune cells. The immune system of fish is extremely well developed [24], comprising both innate [25] and adaptative responses [26]. Fish leukocytes, comprising macrophages, dendritic cells, neutrophilic granulocytes, and lymphocytes, are able to colonize foreign elements invading the host organism [15]. In the present study, we showed that the rainbow trout leukocytes (from head kidney or spleen) reduced motility and induced the *A. simplex* larvae to roll up into spirals. The parasites may have coiled in order to reduce the surface available for the colonization of immune cells, which produce various antiparasitic immune molecules. At the very least, we cannot exclude the possibility that nematodes coil up into a spiral and aggregate to protect themselves (by reducing the total surface area available for binding of immune cells and their effector molecules). In all cases, this process will this serve as an adaptation to the long-lived hypobiotic stage of the worm larva in the paratenic fish host, awaiting a cetacean to ingest the third-stage larva. The larva can then reactivate and fulfil its life cycle by achieving the reproductive stage in the stomach of the whale.

We have used an in vitro model to elucidate to what extent some central abiotic and biotic factors can induce behavioural changes in a parasitic worm. This approach may be applied in future studies in order to investigate other host–parasite models and to include additional environmental factors (internal and external), which were not investigated here.

5. Conclusions

A range of abiotic and biotic factors, such as temperature, pH, electric currents, and immune cells, influence the behaviour of third-stage larvae of the parasitic nematode

A. simplex. The responsiveness of the worm is interpreted as an adaptation to the different conditions it encounters at different life cycle stages. In the mammalian host stomach, the nematode larva moults twice, becomes an adult, and copulates, and female specimens produce eggs. In this study, we saw the highest activity at 37 °C and pH 2, conditions found in the stomach of the definitive host.

In paratenic hosts, the nematode larva often faces lower temperatures (dependent on the seawater temperature) and higher pH (6–9) values. *A. simplex* larvae are not able to moult in the paratenic host, but its reactions to electric and immunological stimuli allow them to attain a long-lived hypobiotic stage in the fish host. This adaptation elevates the probability that the parasite will be ingested by a marine mammal (the definitive host), whereby it can fulfil its life cycle successfully.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/jmse12091546/s1: Table S1. Behaviour of A. simplex nematode larvae when exposed to combinations of electric current and pH. Table S2. Activity level, comparison between different pH conditions at different temperature levels. Table S3. Activity level, comparison between different temperatures at different pH levels. Table S4. Exposure of worms to rainbow trout immune cells from spleen or head kidney.

Author Contributions: Conceptualization, K.B., K.K. and C.M.F.G.; methodology, K.K, C.M.F.G., P.W.K. and K.B.; validation, K.K, C.M.F.G., P.W.K. and K.B.; formal analysis, K.K, C.M.F.G., P.W.K. and K.B.; investigation, K.K, C.M.F.G., P.W.K. and K.B.; resources, K.B. and P.W.K.; data curation, K.K, C.M.F.G., P.W.K. and K.B.; writing—original draft preparation, K.K. and K.B.; writing—review and editing, K.K, C.M.F.G., P.W.K. and K.B.; visualization, K.K, C.M.F.G., P.W.K. and K.B.; supervision, K.B. and P.W.K.; project administration, K.B.; funding acquisition, K.B. All authors have read and agreed to the published version of the manuscript.

Funding: The present study was supported by the Danish Ministry for Food, Agriculture and Fisheries with "OPTIKVAL" grant 34009-22-2100 under the GUDP programme.

Institutional Review Board Statement: Rainbow trout cells were harvested under licence 2024-15-0201-01694 issued by the Experimental Animal Inspectorate. The project was reviewed by the Animal Ethical Institutional Review Board of the University of Copenhagen.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials; further inquiries can be directed to the corresponding author. The data presented in this study are available on reasonable request from the corresponding author.

Acknowledgments: The authors are indebted to the company Scandic Pelagic, Skagen, Denmark, for providing herring for this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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