

Prace oryginalne

Effect of temperature, NaCl concentration and aeration of solutions on the survivability of II stage larvae of *Contracaecum rudolphii* Hartwich, 1964

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ABSTRACT. Determinations were carried out for the effect of temperature, NaCl concentration and aeration of solutions on the survivability of stage II larvae of *Contracaecum rudolphii* nematode. Stage II larvae hatched from egg shells were placed in tap water as well as in 1% and 3% solutions of NaCl. Part of the culture was run on Petri dishes and aerated each day. The second part was transferred into high beakers and left non-aerated. All cultures were run in three replications at temperatures of 4, 10, 20 and 30°C. Microscopic observations of the survivability and activity of the larvae were conducted every day in all samples. The larvae incubated for 30 days at a temperature of 4°C were viable and non-motile. The longest life span was noted in the case of the larvae incubated in the aerated 1% solution of NaCl at a temperature of 10°C, which also recorded the lowest mobility. The shortest life span was reported for the larvae incubated in the non-aerated 3% solution of NaCl at a temperature of 30°C. The results obtained in this study indicate that the II stage larvae of *C. rudolphii* may live both in both fresh and salt waters and a significant factor determining their life span is water saturation with oxygen.

Key words: Nematoda, *C. rudolphii*, larvae, survival

Introduction

The final host of *Contracaecum rudolphii* are ichthyophagous birds, including mainly cormorants (*Phalacrocorax carbo*, *Ph. aristotelis*) [1–7]. In Poland, this nematode was detected for the first time in 1956 in grebe (*Podiceps cristatus*, *P. griseigena*, *P. ruficollis*) and in little grebe (*Tachybaptus ruficollis*) [8], whereas in cormorants it was detected in 2000 [9]. Further investigations have shown that both the extensity and intensity of infections of cormorants breeding in Poland are very high [6,7].

The life cycle of *C. rudolphii* includes two intermediate hosts, the first being Crustacea (Copepoda, Amphipoda) or larvae of aquatic insects, whereas the second intermediate or paratenic host are fish [10–14].

The high extent and intensity of cormorant infections, as well as the hatching season in Poland would indicate a more frequent occurrence of *C. rudolphii* larvae in fish as their source of food. However, in 2007 Szostakowska and Fagerholm first found nematode larvae in crucian carps (*Carassius carassius*) and round gobies (*Neogobius melanostomus*) in Poland [15]. The extent of the infection in both of these species was very low and reached 2.4%. Larvae of *C. rudolphii* are also often detected in fish caught in Chile [3,16], the Black Sea and the Sea of Azov [17–19]. For this reason, it may be speculated that this parasite is developing in waters warmer than those in Poland and that cormorants are becoming infected with this parasite during the wintering season.

Eggs of *C. rudolphii* are classified as "thin-shells" eggs, and during embryonic development they display high susceptibility to temperature, humidity, water oxygenation and such chemical

substances as formalin or a digestive fluid containing pepsin and HCl [10,20,21]. They have been reported to develop both in fresh and sea water. The available literature lacks references on the effect of such abiotic factors as temperature, water salinity and oxygenation of the environment on the survivability of stage II larvae hatching from egg shells. According to a number of authors, the abiotic factors having the most significant impact on the development, survivability and transmission of parasite include temperature [22] and – in the case of the nematodes belonging to the family Anisakidae – also water salinity [23].

In the moderate climatic zone, dimictic water reservoirs are prevalent and are characterized by spring and autumn circulation as well as summer and winter stagnation. The temperature of water of the Mazurian lakes in the epilimnion ranges from 18 to 22°C, and in the hypolimnion from 6 to 9°C. During winter stagnation, the temperature of demersal water is 2.5–4°C. The spring and autumn circulations result in thermal equalization of the whole water reservoir. In the early spring, the temperature of water reaches ca. 4°C, whereas the mean temperature of water in the late spring and early autumn is ca. 10°C [24]. In the Baltic Sea, the water temperature ranges from 0 to 2°C in the winter and from 12 to 22°C in the summer season.

An element determining the biotic conditions of organisms in water reservoirs is oxygen. Lake saturation with oxygen is affected by thermal diversity in the particular strata of the lake, and is almost equal only in the period of spring and autumn circulation. In the period of winter stagnation, the highest quantity of dissolved oxygen will be available just under the surface of the ice. During the summer stagnation in the epilimnion, characterized by a higher temperature, the concentration of oxygen should be lower and should increase in the metalimnion, to reach the highest value in the hypolimnion. However, a high content of oxygen in the demersal strata is typical only of oligotrophic lakes, whereas in eutrophic and dystrophic lakes the content of oxygen at the bottom is very low or null [25].

The objective of the present study was to investigate, under laboratory conditions, the effect of water temperature and oxygenation on the survivability of stage II larvae of *C. rudolphii*. The temperatures tested correspond to the mean temperature of water in the subsequent seasons of the year in water reservoirs located in Poland. The results obtained would demonstrate whether nematode larvae hatching from egg thecas may survive in Polish water reservoirs all year round or only in the summer, and whether their survivability is affected by water oxygenation. For a considerable number of cormorants with their hatching season in Poland, the feeding reservoir is the Baltic Sea. This experiment would demonstrate the feasibility of larvae development in water with similar salinity to the coastal zone of the Baltic Sea (0.2–1.2%). Additional analyses will be carried out in order to verify the survivability of nematode larvae in water with a temperature and salinity similar to the Mediterranean Sea and the Sea of Azov, as nematode larvae are detected relatively frequently in fish in those water reservoirs.

Material and methods

Adult females of *C. rudolphii* were isolated from the stomachs of cormorants obtained from a bird colony in the area of Ełk (northeastern Poland). Until stage II larvae were obtained, the eggs were incubated in tap water and in 1% and 3% solutions of NaCl at room temperature. Larvae of each solution were separated from egg shells using the method of Baermann [26], by placing a thin layer of cotton-wool at the bottom of a funnel. Cultures of the larvae (approx. 5000 larvae in each culture) were run in tap water as well as in 1% and 3% solutions of NaCl on Petri dishes or in high beakers, at temperatures of 4, 10, 20 and 30°C, in three replications. All the cultures run on the dishes were aerated daily by rapidly filling and emptying a pipette with an ejector. The solutions in all cultures were changed every second day. The survivability and motility of the larvae were determined daily in all solutions by placing containers with the larvae under a binocular and assaying the percentage of viable larvae. The motility of the larvae was assessed on a 4-degree scale: very motile larvae (+++), motile larvae (++), poorly motile larvae (+) and non-motile (–).

Results

Results referring to the survivability and motility of the larvae in particular solutions are provided in Table 1.

The larvae incubated at a temperature of 4°C were non-motile, they started to move after 30 min at a room temperature. When the temperature of

Table 1. The effect of temperature, NaCl concentration and aeration of solutions on the survivability [%] and motility of stage II larvae of *Contracaecum rudolphii* Hartwich, 1964

Mean % of live larvae and standard deviation							
		Tap water		1% NaCl		3% NaCl	
°C	Days	A	B	A	B	A	B
4	1–30	–	–	–	–	–	–
10	2	100 ++	80.00±4.90 ++	100 ++	85.33±8.65 ++	100 ++	75±5.72 ++
	5	100 ++	51.88±11.52 +	100 ++	55.25±9.50 +	100 ++	48±12.39 +
	7	75.88±7.68 ++	–	85.50±10.32 ++	14.75±5.26 +	64.13±9.06 ++	4.75±3.20 +
	14	50.90±9.81 +	–	66.60±9.36 ++	–	48.00±10.22 +	–
	20	–	–	46.63±5.76 ++	–	–	–
20	2	100 +++	74.67±6.66 +++	100 +++	84.67±4.16 +++	100 +++	65.00±4.36 ++
	3	100 +++	52.00±3.61 ++	100 +++	56.33±12.22 +++	100 +++	44.67±4.62 +++
	7	88.25±8.28 +++	31.88±5.36 +	92.63±5.63 +++	34.88±6.31 ++	85.75±10.57 ++	23.88±7.79 ++
	14	49.40±0.74 +++	5.20±3.12 +	55.60±11.09 +++	9.70±5.40 +	45.80±5.05 ++	2.10±2.08 +
30	2	100 +++	67.33±7.04 +++	100 +++	78.67±6.94 +++	100 +++	45.00±5.10 +
	3	94.33±5.03 +++	49.33±7.51 +	96.67±1.53 +++	51.00±2.65 ++	79.67±9.02 +	19.67±10.02 +
	7	78.90±12.18 +++	34.70±4.64 +	89.60±11.54 +++	38.70±4.88 ++	47.60±6.04 ++	–
	10	48.30±10.97 +++	2.20±2.15 +	61.40±17.58 +++	5.00±2.98 +	27.60±10.65 +	–
	14	20.20±10.98 +	–	31.80±10.20 +	–	–	–

Explanations: A–aerated cultures; B–non-aerated cultures;+++ very motile larvae; ++ motile larvae; + poorly motile larvae; – non-motile larvae

their incubation was increased to 20°C after 30 days, they demonstrated the same motility and survivability as the larvae kept at that temperature from the beginning of incubation.

In the aerated cultures run at a temperature of 10°C, all larvae were viable and motile until day 5 of incubation. In the consecutive days, the number of live and motile larvae decreased successively. On day 14 of the incubation, the percentage of live, motile larvae reached ca. 50%. The longest life span was noted in the case of the larvae incubated in the aerated 1% solution of NaCl. In the non-aerated cultures, on the second day of incubation the percentage of viable larvae accounted for 80%, whereas on the fifth day it accounted for ca. 50%. On day 7, there were no motile larvae in tap water, whereas in the 1% solution of NaCl the percentage of viable larvae reached ca. 15% and 5% in the 3% NaCl solution.

In the cultures run at temperatures of 20 and 30°C, the larvae were more motile but had shorter life spans than those incubated at a temperature of 10°C. On day 14 of incubation, the percentage of viable larvae accounted for ca. 50% in the aerated cultures run at a temperature of 20°C. On the following day, the number of live larvae dramatically decreased. In the cultures run in tap water and 1% NaCl at a temperature of 30°C, the percentage of live larvae reached ca. 50% on day 10 of incubation, whereas in the culture run in 3% NaCl the same percentage of live larvae was noted on day 7 of incubation. On consecutive days of

incubation, the number of viable larvae was observed to decrease rapidly.

In the non-aerated cultures, on the third day of incubation the percentage of viable larvae accounted for ca. 50%. The shortest life span was reported for the larvae kept in the non-aerated 3% solution of NaCl and incubated at a temperature of 30°C. On the second day of incubation in that medium, the percentage of viable larvae reached only 45%, whereas on the third day it was only 19.67%.

Since they had hatched from the egg shells, the larvae in all cultures remained inside the cuticular sheath throughout the culture period.

Discussion

Embryonic development of most of nematodes proceeds in the natural environment. Eggs of soil nematodes, the so-called "geohelminths", are coated with a thick, three-layer shells that protects them against detrimental environmental factors. The hatching of larvae occurs only when eggs of the parasite are transferred into the final host and are stimulated by specific physicochemical factors, including: temperature, pH and CO_2 [27]. In the case of the so-called "thin-shells" eggs, the "spontaneous release of larvae" occurs before they reach the host. At the moment of hatching, the larvae are exposed to environmental factors, therefore they often keep the cuticula from the previous molt as a "protective sheath", which they loose in the gastrointestinal tract of the host. In the case of some parasites, the hatching of larvae is significantly affected by access to light, salinity and temperature of water [23]. The current study, as well as studies by other authors demonstrated that the rate of *C. rudolphii* egg development to the larval stage was determined by such environmental factors as temperature, oxygen saturation and salinity of water [10,14,20,21]. In aerated tap water at 23°C the larval stage development spans 6 days, whereas at lower temperatures it spans 14 days. The stage II larvae releasing spontaneously from egg shells keep the sheath from the previous molt [10,11, 13,14].

There is a lack of available research in literature on the effect of environmental factors on the hatching of larva. The results of the presented experiment demonstrate that the survivability of stage II larvae of *C. rudolphii* is affected by both temperature, salinity and oxygen saturation of water. Complete inhibition of the motility of larvae occurred at 4°C, but did not result in death. When 30 days later they were transferred to a temperature of 20°C, they displayed a similar motility and survivability as the larvae which were kept at the same temperature immediately after hatching. These results may indicate that the larvae reaching hypolimnion (where the temperature of water is ca. 4°C) before stagnation may survive and constitute the parasite's reservoir. In the springtime, during spring circulation they reach warmer surface waters, become active and find their hosts. Nevertheless, residing for too long at a low temperature affects negatively their invasiveness. Copepods have been shown to remain uninfected with larvae of *Pseudoterranova decipiens* stored for 120 days at a temperature of 0–5°C [28]. The low temperatures may also arrest larvae transfer from intermediate hosts to final hosts [29], which affects the lower prevalence of the larvae in fish of cold waters. The complete elimination of larvae from the lake ecosystem may occur only in shallow water reservoirs or in the coastal zone of deep reservoirs where water freezes over.

The larvae incubated at temperatures of 20 and 30°C appeared to be more motile than those incubated at a temperature of 10°C, but their life span was still considerably shorter.

Nematodes are heterothermic animals whose reserves of energy are utilized more intensively with a temperature increase. After hatching, the stage II larvae are covered with a "sheath" from the previous moulting, which prevents additional feeding and limits the larvae to reserves accumulated in the egg. The results obtained show that the cooler water in the Polish water reservoirs in the spring and autumn seasons (ca. 10°C) may have a positive impact on the transmission of the nematode. This period is additionally characterized by a high availability of potential intermediate hosts (Copepoda). In contrast, in the summer season when the water temperature is higher, the larvae display a higher motility, however, with a lack of intermediate hosts this may negatively affect the transmission of the nematode in the ecosystem.

Similar results were obtained when assessing the effect of temperature on the survivability of *Anisakis simplex* and *P. decipiens* larvae [23,30,31]. According to a number of authors, of all the abiotic factors, temperature has the greatest impact on the development and transmission of parasites [22,28].

The second factor thought to have a considerable effect on the survivability of *C. rudolphii* larvae is

the salinity of water. In the current study, irrespective of the incubation temperature, the presence of sodium and chloride ions did not exert any significant effect on the life span of the larvae, with the longest life span and motility being reported for the larvae incubated in the aerated 1% solution of NaCl, and the shortest for the larvae incubated in 3% NaCl. In an experiment by Huizinga [10], the II stage larvae kept in sea water (the author does not provide water salinity) lived from 30 days to 6 months, depending on the water temperature, whereas those hatched in sea water and transferred into tap water swelled and died within a few days, indicating that the larvae of *C. rudolphii* preferred salty water. Perhaps it was the change in water salinity that affected osmoregulation and evoked such differences in the life span of the larvae. In the reported experiment, the larvae were kept in the solutions which the embryonic development proceeded in. No problems with osmoregulation were observed in any of the solutions. The authors' previous findings, as well as results of this study indicate that this parasite may develop successively in both fresh and salty waters, and that the larvae hatching from egg shells develop protective mechanisms against excessive hydration and dehydration.

The larvae of other Anisakidae (*P. decipiens*, *A. simplex*) prefer saline water. Irrespective of temperature, larvae of *A. simplex* were reported to have the longest life span in water with 28‰ salinity, whereas larvae of *P. decipiens* had the longest life span in water with 35‰ salinity [23,31].

A factor that distinctively reduced the survivability of *C. rudolphii* larvae in the reported experiment was the oxygenation of the solutions. Irrespective of temperature and salinity, the larvae had a substantially shorter life span in the non-aerated solutions, as compared to the aerated ones. Especially distinct differences in the life span of the larvae were observed in solutions with a temperature of 30°C, in which on the third day of incubation the percentage of active larvae accounted for as low as ca. 20%. The available literature lacks reports on the effect of this factor on the survivability of larvae of other Anisakidae. The likely cause of such a rapid mortality of the larvae in the non-aerated solutions was the limited concentration of oxygen. Developmental stages of a number of parasites (egg, miracidium, cercaria, larvae of nematodes) display mainly aerobic metabolism, which is considerably more efficient than anaerobic metabolism.

In Poland, the best (and almost equal) water oxygenation in water reservoirs occurs in the period of spring and autumn circulation. In turn, in the summer – when the temperature of water reaches over 20°C – the content of dissolved oxygen in the epilimnion is substantially lower. In the case of oligotrophic lakes, the highest content of oxygen in this period is reported in the hypolimnion. However, most lakes in Poland are classified as eutrophic or dystrophic lakes, in which the content of oxygen in the hypolimnion in the summer time is low or null [24]. In these types of lakes, the best conditions for larvae development occur in the coastal zone.

The results obtained in this study indicate that the II stage larvae of *C. rudolphii* may live both in both fresh and salt waters, and that a factor having a significant impact on their life span is water saturation with oxygen. The high extent and intensity of cormorant infections [6,7], e.g. with nematode, as well as results of the reported experiment are likely to indicate the prevalence of *C. rudolphii* larvae in fresh and salt waters and point to the possibility of fish infections. The longest life span in the present experiment (irrespective of temperature) was reported for the larvae incubated in the aerated 1% solution of NaCl. This corresponds to the water conditions of Gdańsk Bay in the spring and summer season, i.e. when cormorants inhabit the bay. Previous long-term studies of fish originating from water reservoirs located close to a cormorant colony in Kąty Rybackie and at Lake Wulpińskie did not demonstrate the presence of nematode larvae in the examined fish [32,33]. It was not until 2007 that Szostakowska and Fagerholm [15] were the first to detect nematode larvae in fish from Poland. Out of 10 fish species analyzed by those authors (*Carassius carassius*, *Scardinius erythrophthalmus*, *Abramis brama*, *Blicca björkna*, *Tinca tinca*, *Rutilus rutilus*, *Esox lucius*, *Lucioperca lucioperca*, *Acerina cernua*, *Neogobius melanostomus*), only Crucian carps from Lake Selment Wielki and round gobies from Gdańsk Bay were shown to contain the larvae of *C. rudolphii*. The extent and intensity of infections was very low.

One of the reasons for the rare occurrence of *C. rudolphii* larvae in Polish fish may be the etiological differences between larvae of nematodes and the intermediate host (a closed etiological filter) [34]. The first intermediate host of *C. rudolphii* are copepods [10–14]. In the summer season, the

temperature of water facilitates the development of *C. rudolphii* eggs and the activity of larvae; in that period, the number of copepods in zooplankton is low. Lower water temperatures in the autumn and spring seasons facilitate a higher number of copepods, but affect a decrease in the number of hatched larvae and diminish their activity. Studies into the role of copepods in the life cycle of *C. rudolphii* have shown that once they are eliminated, the intensity and extent of fish infections are very low [10,13,35]

The temporary inhibition of life activity of copepods, caused by cyclic unfavorable conditions occurring in the environment (diapause), may significantly affect the transmission of a number of fish parasites [36]. For this reason, the larvae of *C. rudolphii* are detected considerably more frequently in fish from waters characterized by a more stable temperature [3,16–19].

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