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Original Article

# *In vitro* culture and morphology of *Neoechinorhynchus buttnerae* (Eoacanthocephala: Neoechinorhynchidae) collected from the intestine of tambaqui (*Colossoma macropomum*) farmed in the Brazilian Amazon

## Cultivo *in vitro* e morfologia de *Neoechinorhynchus buttnerae* (Eoacanthocephala: Neoechinorhynchidae) coletados do intestino de tambaqui (*Colossoma macropomum*) cultivado na Amazônia brasileira

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**Abstract**

The tambaqui (*Colossoma macropomum*) is a species of great economic importance for fish farming in the Brazilian Amazon, and acanthocephaliasis caused by *Neoechinorhynchus buttnerae* (Golvan 1956) represents an obstacle to its production due to it causing severe morphological damage to the intestinal mucosa, thus impairing the absorption of nutrients and causing weight loss in the fish. Therefore, the establishment of *in vitro* protocols for evaluation of anthelmintic drugs is the first step to development of effective measures for *in vivo* control of this endoparasite. The present study evaluated the *in vitro* survival of *N. buttnerae* maintained in Eagle's minimum essential medium under different culture conditions. Three assays were carried out to evaluate whether temperature, supplementation with the antibiotics penicillin and streptomycin, and culture medium replacement or no replacement would influence the motility and morphology of the acanthocephalans. The results of the Kaplan-Meier analysis indicated that the use of culture in minimum essential medium together with penicillin and streptomycin prolonged the parasite's survival when kept at temperatures of 24 °C or 28 °C. We describe herein for first time an alternative protocol that is ideal for the *in vitro* culture of *N. buttnerae*. As such, this protocol ensures greater reliability in further *in vitro* studies with *N. buttnerae*.

**Keywords:** acanthocephalan, culture medium, endoparasite, viability.

**Resumo**

O tambaqui (*Colossoma macropomum*) é uma espécie de grande importância econômica para a piscicultura na Amazônia brasileira, e a acantocefalose causada por *Neoechinorhynchus buttnerae* (Golvan 1956) representa um obstáculo à sua produção por causar severos danos morfológicos à mucosa intestinal, prejudicando a absorção de nutrientes e causando perda de peso nos peixes. Assim, o estabelecimento de protocolos *in vitro* para avaliação de fármacos anti-helmínticos é o primeiro passo para o desenvolvimento de medidas eficazes de controle *in vivo* destes endoparasitas. O presente estudo avaliou a sobrevivência *in vitro* de *N. buttnerae* mantido em meio essencial mínimo de Eagle sob diferentes condições de cultivo. Três ensaios foram realizados para avaliar se a temperatura, a suplementação com os antibióticos penicilina e estreptomicina e a substituição ou não do meio nutriente influenciariam a motilidade e a morfologia dos acantocéfalos. Os resultados da análise de Kaplan-Meier indicaram que o uso do meio essencial mínimo suplementado com penicilina e estreptomicina prolongou a sobrevivência do parasito quando mantido em temperaturas de 24 °C ou 28 °C. Este estudo descreve pela primeira vez um protocolo alternativo que é ideal para o cultivo *in vitro* de *N. buttnerae*. Este protocolo garante maior confiabilidade em ensaios *in vitro* com *N. buttnerae*.

**Palavras-chave:** acantocéfalo, meio de cultivo, endoparasito, viabilidade.

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## 1. Introduction

Acanthocephaliasis caused by *Neoechinorhynchus buttnerae* (Golvan 1956) represents a significant problem in fish farming in the Brazilian Amazon (Silva-Gomes et al., 2017; Pereira and Morey, 2018; Valladão et al., 2020), and compromises production and productivity (Silva-Gomes et al., 2017). This acanthocephalan occurs mainly in farmed *Colossoma macropomum*, which is the most produced native fish species in the region (Hilsdorf et al., 2022). In high *N. buttnerae* infection rates, the host fish suffer severe morphological intestinal damage and, upon reaching the villi, this endoparasite impairs the absorption of nutrients, thus compromising the growth of the host (Matos et al., 2017).

In order to establish protocols for the control and treatment of infections by *N. buttnerae*, *in vitro* studies with anthelmintic drugs are paramount. These aim to select drugs with high efficacy in promoting parasite mortality after a certain period of exposure. However, for the proper performance of these tests, it is necessary to guarantee the viability of parasites, which, when outside the host, suffer from the influence of external factors. As such, it is necessary to meet their nutritional requirements in order to guarantee their survival (Ahmed, 2014; Scare et al., 2019). Therefore, choosing the best nutrient medium for parasite maintenance is an essential step (Sangmaneedet and Smith, 2000). Chemically defined media containing amino acids, vitamins and other nutrients have been used for the maintenance or cultivation of helminth parasites *in vitro* (Ahmed, 2014). Moreover, supplementation with antibiotics in the maintenance media and the renewal of the media (Hamers et al., 1991; Costa et al., 2018; Niciura et al., 2023) provide optimal conditions for maintaining helminth viability for long periods (Yasuraoka and Hata, 2003; Niciura et al., 2023).

Different culture mediums have been successfully used in the *in vitro* culture of cestodes (Bucur et al., 2019), trematodes (Hardy-Smith et al., 2012; Uddin et al., 2012), nematodes (Njouendou et al., 2017; Heredia et al., 2018; Zofou et al., 2018; Scare et al., 2019) and acanthocephalans (Harms, 1965; Taraschewski et al., 1990). However, there are few studies regarding the viability of *in vitro* culture of *N. buttnerae* (Valladão et al., 2020). Thus, considering this gap in information regarding methods, the present study aimed to determine the *in vitro* survival time of *N. buttnerae* in Eagle's minimum essential medium supplemented with the antibiotics penicillin and streptomycin, as well as under different temperatures.

## 2. Material and methods

### *2.1. Acclimation of fish and acquisition of parasites*

*Colossoma macropomum* fingerlings (19.3 ± 0.12 cm and 268.7 ± 6.2 g) naturally infected by *N. buttnerae* (mean intensity of 634.1 ± 129.7) were acquired from a fish farm with a known history of the occurrence of acanthocephalosis and then transported to the fish farming sector of Embrapa Amazônia Ocidental (Amazonas state, Brazil), where they were kept in 1,000 L tanks, with constant aeration and heating, for 30 days. During this period, the fish were fed twice a day with commercial feed containing 32% crude protein until apparent satiety. Fish naturally infected by *N. buttnerae* adults were used in all trials.

During this period, the following water quality parameters were monitored: dissolved oxygen (5.2 ± 0.04 mg $L^{-1}$) and temperature (28.1 ± 0.04 °C), measured with digital oximeter (YSI Pro20, YSI Inc., USA), pH (5.8 ± 0.23) using digital pH meter (YSI F-1100, YSI Inc., USA), alkalinity (4.9 ± 0.3 mg $L^{-1}$) and hardness (8.6 ± 0.5 mg $L^{-1}$) using titrimetry, and total ammonia (0.7 ± 0.03 mg $L^{-1}$) by the indophenol method, according to APHA (1998), and did not differ between treatments.

The study was approved by the Animal Use Ethics Committee at Embrapa Amazônia Ocidental (Protocol No. 03/2018), and access to genetic heritage (fish and parasites) was regularized through the authorization No. AB1F0FA, obtained from the Genetic Heritage Management Council (CGEN), Ministry of the Environment (MMA).

### *2.2. Viability assays of Neoechinorhynchus buttnerae*

After anesthesia with benzocaine (250 mg $L^{-1}$) and euthanasia of fish by medullar section (according to the National Council for Animal Experimentation Control - CONCEA guidelines; Brasil, 2018), the intestines were removed and dissected for removal of *N. buttnerae* adults, which were then transferred to Petri dishes containing saline (0.9%) and, subsequently, visualized under a stereomicroscope (Leica, EZ4).

Live *N. buttnerae* adults were washed twice with PBS (phosphate buffered saline) and used for the assays as described below. For this study, three treatments were performed. In the first, 5 mL of Eagle's minimum essential medium (EMEM) (Sigma-Aldrich, UK) at pH 7.2 was used, and the Petri dishes with the parasites were placed in an incubator (Tecnal TE 401) at 24, 26, 28, 30 and 32 °C. In the second, 5 mL of EMEM at pH 7.2, supplemented with 100 U $mL^{-1}$ penicillin (Life Technologies) and 100 µg $mL^{-1}$ streptomycin (Life Technologies) (Hamers et al., 1991), was used, and the Petri dishes with the parasites were incubated at 24, 26, 28, 30 and 32 °C. In the third, 5 mL of EMEM at pH 7.2, supplemented with 100 U $mL^{-1}$ penicillin and 100 µg $mL^{-1}$ streptomycin was used, and the Petri dishes with the parasites were incubated at 24, 26, 28, 30 and 32 °C, and every two days the EMEM and antibiotics were removed and replaced with freshly prepared media. In all treatments, 10 parasites in each one of three triplicates (30 parasites by treatment) were used. The samples were manipulated in a laminar flow cabinet and the media was filtered using PES membranes (0.22 µm) to ensure sterile conditions.

Survival analysis was performed every day until all the parasites died. Parasites were considered dead when they presented absence of any motility, even after stimulation, and were discarded. The parasites were considered alive when they presented fast or slow movements of any part of the body or the whole body, discrete movements of the proboscis or body fluids, and/or movement of internal organs. In order to associate morphological descriptors with changes in motility over time, macroscopic changes in the integument, in the coloration, swelling or dehydration of

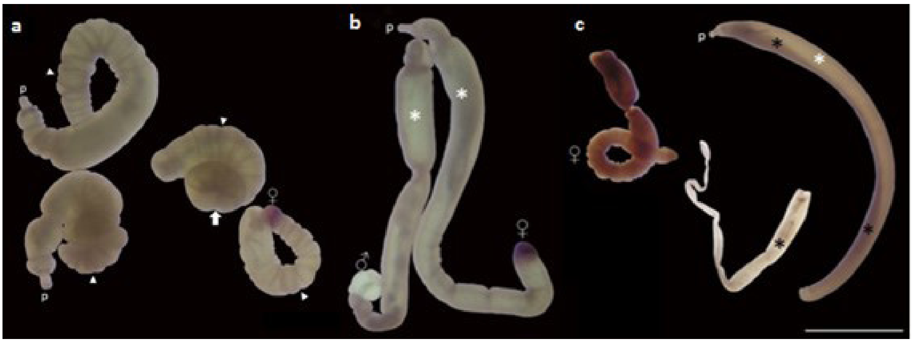

**Figure 1.** Morphological patterns of *Neoechinorhynchus buttnerae* maintained in Eagle`s minimum essential medium and with antibiotics. (a) Live parasites with coiling of the posterior portion of the metasoma (arrow), characteristic wrinkling of the integument (arrowheads) and transparency of the body with easy visualization of the pseudocoelom and internal organs; (b) Moribund parasites with body swelling (*), loss of integument wrinkling, without proboscis eversion (p) even under stimulation; (c) Dead, blackened and distended parasites, with body swelling (white asterisk) and loss of integument wrinkling, with deterioration of internal organs (black asterisk) or deformed, dehydrated and friable in deteriorating (arrows). Bar: 1 cm.

the body, deterioration of internal organs and loss of body integrity were recorded throughout the trials.

*2.3. Statistical analysis*

The survival period (days) was determined from the beginning of the experiments until the occurrence of death of each parasite. This factor was used in the Kaplan-Meier survival analysis. The survival curves were obtained for each assay and also between the treatments. Results were statistically compared using the log-rank test and were considered significant when $p < 0.001$. The statistical analysis was performed using Prism, version 5.0 (Prism Software, Irvine, CA, USA).

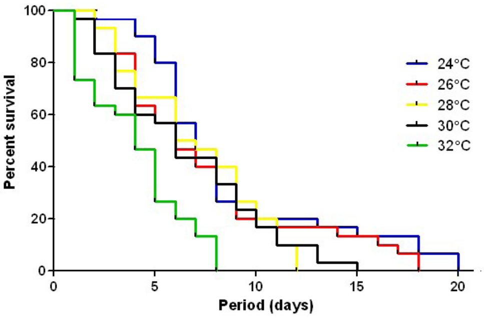


**Figure 2.** Kaplan-Meier estimated survival curves for *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium with different temperatures and without supplementation with antibiotics and without culture medium renewal.

## 3. Results

*3.1. Viability of Neoechinorhynchus buttnerae: motility and morphology*

For *N. buttnerae*, three different morphological and motility patterns were observed: 1) parasites with twisting of the posterior portion of the metasome, with characteristic wrinkling of the integument and transparency of the body with easy visualization of the pseudocoelom and internal organs, especially the reproductive organs (Figure 1a). These showed rapid movements of the whole body, or parts of the body, eversion of the proboscis or movements of fluids in the pseudocoelom, naturally or under slight stimulation. 2) Partially or totally swollen parasites with partial or total distension of the body and loss of wrinkling in the integument (Figure 1b). In this case, slow movements of parts of the body only under stimulation and absence of eversion of the proboscis were observed. 3) Parasites were darkened, totally distended, swollen, with loss of wrinkling in the integument, or dehydrated and friable, with partial or total deterioration of internal organs, or deterioration of the whole organism (Figure 1c). In this case, the absence of motility was observed, even under stimuli. Based on these descriptors, the parasites observed in pattern 3 were considered unviable.

*3.2. Survival of Neoechinorhynchus buttnerae under different maintenance conditions and temperatures*

In parasites maintained with Eagle's minimum essential medium and incubated at 24, 26, 28, 30 and 32 °C, the maximum survival time was 20 days (Figure 2). Similar survival time was observed in parasites maintained with Eagle's minimum essential medium and antibiotics without culture medium replacement (Figure 3). However, when Eagle's minimum essential medium was supplemented with antibiotics and replaced every two days, the parasites' maximum survival time increased to 42 days (Figure 4).

For parasites maintained with Eagle's minimum essential medium, the range of mean survival time (the time in which the survival fraction equals 50%) was from 4 to 7 days. At temperatures of 24 °C, 26 °C, and 28 °C, all acanthocephalans survived two days. At temperatures of 30 °C and 32 °C, this time was 24 h and less than 24 h, respectively (Table 1). In this assay, the survival percentage of *N. buttnerae* did not differ over time within the temperature range of 24-30°C ($p>0.001$), and the survival period was significantly longer when compared to the temperature of 32 °C (Table 1).

**Table 1.** Median, maximum and 100% survival period of *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium, without antibiotics and without culture medium replacement.

| Temperature | N | Median survival time (days) | Maximum survival time (days) | Time (days) of 100% survival | Tested temperatures | Critical values for the qui-square distribution ($\chi 2$) | p |
|---|---|---|---|---|---|---|---|
| 24 °C | 30 | 7 | 18 | 1 | 24 °C vs 26 °C | 1.21 | 0.270 |
| | | | | | 24 °C vs 28 °C | 1.61 | 0.204 |
| 26 °C | 30 | 6 | 17 | 1 | 24 °C vs 30 °C | 20.72 | 0.0001* |
| | | | | | 24 °C vs 32 °C | 18.15 | 0.0001* |
| 28 °C | 30 | 6.5 | 11 | 1 | 26 °C vs 28 °C | 0.31 | 0.579 |
| | | | | | 26 °C vs 30 °C | 0.99 | 0.319 |
| 30 °C | 30 | 6 | 13 | < 24 h | 26 °C vs 32 °C | 11.18 | 0.0008* |
| | | | | | 28 °C vs 30 °C | 0.001 | 0.967 |
| 32 °C | 30 | 4 | 7 | < 24 h | 28 °C vs 32 °C | 14.83 | 0.0001* |
| | | | | | 30 °C vs 32 °C | 10.01 | 0.0016* |

Comparison by log-rank test of survival curves between different temperatures. *Indicates significant difference (p<0.01).

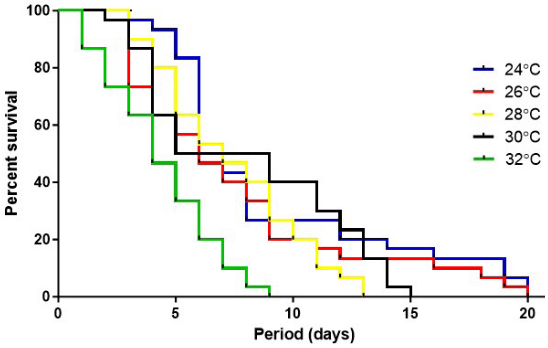


**Figure 3.** Kaplan-Meier estimated survival curves for *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium with antibiotics at different temperatures and without culture medium renewal.

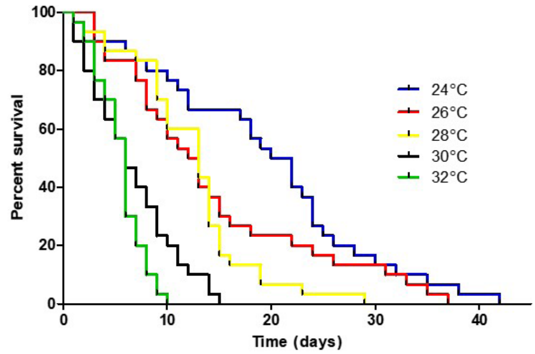


**Figure 4.** Kaplan-Meier estimated survival curves for *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium with antibiotics at different temperatures and culture medium renewal.

For parasites maintained in Eagle's minimum essential medium with penicillin and streptomycin and incubated at 24 °C, 26 °C, 28 °C, 30 °C and 32 °C, the mean survival time was also 4 to 7 days; however, the survival time for 100% of the acanthocephalans at temperatures of 24 °C and 26 °C increased to two days. At higher temperatures, this survival time was less than 24 h (Table 2). At the lower temperatures of 24 °C and 26 °C, the percentage of survival was significantly higher (p<0.001) than at the highest temperatures tested (30 °C and 32 °C). There was no significant difference (p>0.001) in the survival time of the endoparasites at temperatures of 26 °C and 28 °C (Table 2).

For parasites maintained in Eagle's minimum essential medium with penicillin and streptomycin, incubated at 24 °C, 26 °C, 28 °C, 30 °C, and 32 °C, and with the renewal of the medium every two days, the range of mean survival time varied between 6 and 21 days. The survival time of the all the acanthocephalans at temperatures of 24 °C and 26 °C was two days. At higher temperatures, this survival time was lower than 24 h (Table 3). At the lower temperatures (24 °C and 26 °C), the survival percentage was significantly higher than at the highest temperatures tested (30 °C and 32 °C). There was no significant difference in survival time between the intermediate temperatures (26 °C and 28 °C) (Table 3).

### *3.3. The effect of temperature on survival time of Neoechinorhynchus buttnerae between treatments*

Comparing the negative control without culture medium renewal (AR [--]) with the treatment supplemented with antibiotics and without Eagle's minimum essential medium renewal (AR [+-]), there was no difference in the maximum parasite survival time at any of the tested temperatures (Table 4). Significantly higher values (p<0.001) in survival time were observed when there was both the substitution of the Eagle's minimum essential medium and supplementation with antibiotics (AR [++]), when

**Table 2.** Median, maximum and 100% survival period of *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium, with antibiotics and without culture medium renewal.

| Temperature | N | Median Time (days) | Maximum survival time (days) | Time (days) of 100% survival | Tested temperatures | Critical values for the chi-square distribution ($\chi 2$) | p |
|---|---|---|---|---|---|---|---|
| 24 °C | 30 | 7 | 20 | 2 | 24 °C vs 26 °C | 1.18 | 0.276 |
| | | | | | 24 °C vs 28 °C | 2.35 | 0.126 |
| 26 °C | 30 | 6 | 20 | 2 | 24 °C vs 30 °C | 1.53 | 0.216 |
| | | | | | 24 °C vs 32 °C | 19.15 | <0.0001* |
| 28 °C | 30 | 7 | 13 | 2 | 26 °C vs 28 °C | 0.072 | 0.788 |
| | | | | | 26 °C vs 30 °C | 0.006 | 0.934 |
| 30 °C | 30 | 7 | 15 | 1 | 26 °C vs 32 °C | 10.02 | 0.0015* |
| | | | | | 28 °C vs 30 °C | 2.22 | 0.138 |
| 32 °C | 30 | 4 | 9 | < 24 h | 28 °C vs 32 °C | 15.88 | <0.0001* |
| | | | | | 30 °C vs 32 °C | 14.94 | 0.0001* |

Comparison by log-rank test of survival curves between different temperatures. *Indicates significant difference ($p<0.01$).

**Table 3.** Median, maximum and 100% survival of *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium, with antibiotics and culture medium renewal.

| Temperature | N | Median survival time (days) | Maximum survival time (days) | Time (days) of 100% of survival | Tested temperatures | Critical values for the chi-square distribution ($\chi 2$) | p |
|---|---|---|---|---|---|---|---|
| 24 °C | 30 | 21 | 42 | 2 | 24 °C vs 26 °C | 2.95 | 0.0856 |
| | | | | | 24 °C vs 28 °C | 12.40 | 0.0004* |
| 26 °C | 30 | 12.5 | 34 | 2 | 24 °C vs 30 °C | 34.19 | <0.0001* |
| | | | | | 24 °C vs 32 °C | 41.25 | <0.0001* |
| 28 °C | 30 | 13 | 28 | < 24 h | 26 °C vs 28 °C | 1.112 | 0.2908 |
| | | | | | 26 °C vs 30 °C | 16.53 | <0.0001* |
| 30 °C | 30 | 6 | 16 | < 24 h | 26 °C vs 32 °C | 29.37 | <0.0001* |
| | | | | | 28 °C vs 30 °C | 18.06 | <0.0001* |
| 32 °C | 30 | 6 | 11 | < 24 h | 28 °C vs 32 °C | 3.39 | 0.0656 |

Comparison by log-rank test of survival curves between different temperatures. *Indicates significant difference ($p<0.01$)

**Table 4.** Influence of temperature on the *in vitro* survival of *Neoechinorhynchus buttnerae* in Eagle's minimum essential medium without antibiotics and without culture medium renewal (AR [--]); supplemented with antibiotics and without culture medium renewal (AR [+-]) and supplemented with antibiotics and with culture medium renewal (AR [++]).

| Temperature | Treatments | AR [+-] | AR [++] |
|---|---|---|---|
| 24 °C | AR [--] (18) | $\chi^2$=0.044; p=0.834 (20) | $\chi^2$= 25,03; p <0.0001* (42) |
| 26 °C | AR [--] (17) | $\chi^2$=10.90; p=0.723 (20) | $\chi^2$=10.90; p=0.0010* (34) |
| 28 °C | AR [--] (11) | $\chi^2$=0.2122; p=0.645 (13) | $\chi^2$=24.82; p<0.0001* (28) |
| 30 °C | AR [--] (13) | $\chi^2$=2.399; p=0.1214 (15) | $\chi^2$= 0.0023; p=0.878 (16) |
| 32 °C | AR [--] (07) | $\chi^2$=0.575; p=0.076 (09) | $\chi^2$=4.41; p=0.035 (11) |
| 24 °C | AR [+-] (18) | - | $\chi^2$=23.42; p <0.0001* (42) |
| 26 °C | AR [+-] (17) | - | $\chi^2$= 11.47; p <0.0007* (34) |
| 28 °C | AR [+-] (11) | - | $\chi^2$=22.23; p <0.0001* (28) |
| 30 °C | AR [+-] (13) | - | $\chi^2$=2.39; p=0.1214 (16) |
| 32 °C | AR [+-] (07) | - | $\chi^2$=3.363; p=0.067 (11) |

In brackets, the survival times. *Indicates significant difference ($p<0.01$); $\chi^2$: critical values for the chi-square distribution.

compared to the control, at temperatures of 24 °C, 26 °C, 28 °C and 32 °C (Table 4). At 30 and 32 °C, this percentage of survival did not differ (p>0.001) between treatments (Figure 5). When standardizing the supplementation of the Eagle's minimum essential medium with antibiotics, in the AR [+-] and AR [++] treatments, a significantly higher survival time (p<0.001) was observed at the lower temperature of 24 °C, 26 °C and 28 °C (Figure 5). However, at 30 °C and 32 °C, the survival time was the same (p>0.001) between experimental treatments (Table 4).

## 4. Discussion

*In vitro* culture of helminths and the interpretation of the results should be carried out by quantifying the viability of these parasites. In general, the main parameter evaluated is the visual inspection of body mobility of the parasite over time. Thus, variation in motility has been the parameter used in *in vitro* culture studies of trematode (Uddin et al., 2012), nematode (Chapman et al., 1994; Njouendou et al., 2017; Zofou et al., 2018; Scare et al., 2019) and acanthocephalan survival (Costa et al., 2018). In the present study, in addition to motility, the spiral twisting of the posterior part of the body, the presence of wrinkles in the integument, the integrity of the internal organs and body movements clearly distinguished the living *N. buttnerae* from the dead *N. buttnerae*.

Unviable *N. buttnerae* collected from *C. macropomum* have shown darkened, distended, or dehydrated and friable body swelling, with internal organs or the whole-body deterioration signs, without any motility. These morphological features associated with mortality of the parasites were similar to those described by Costa et al. (2018) for *N. buttnerae* of *C. macropomum* after maintenance in RPMI 1640 and Leibowitz culture medium, in which body swelling and dehydration of parasites also occurred, culminating in the death of the worms. Dunagan (1962) also reported that acanthocephalans, regardless of the length

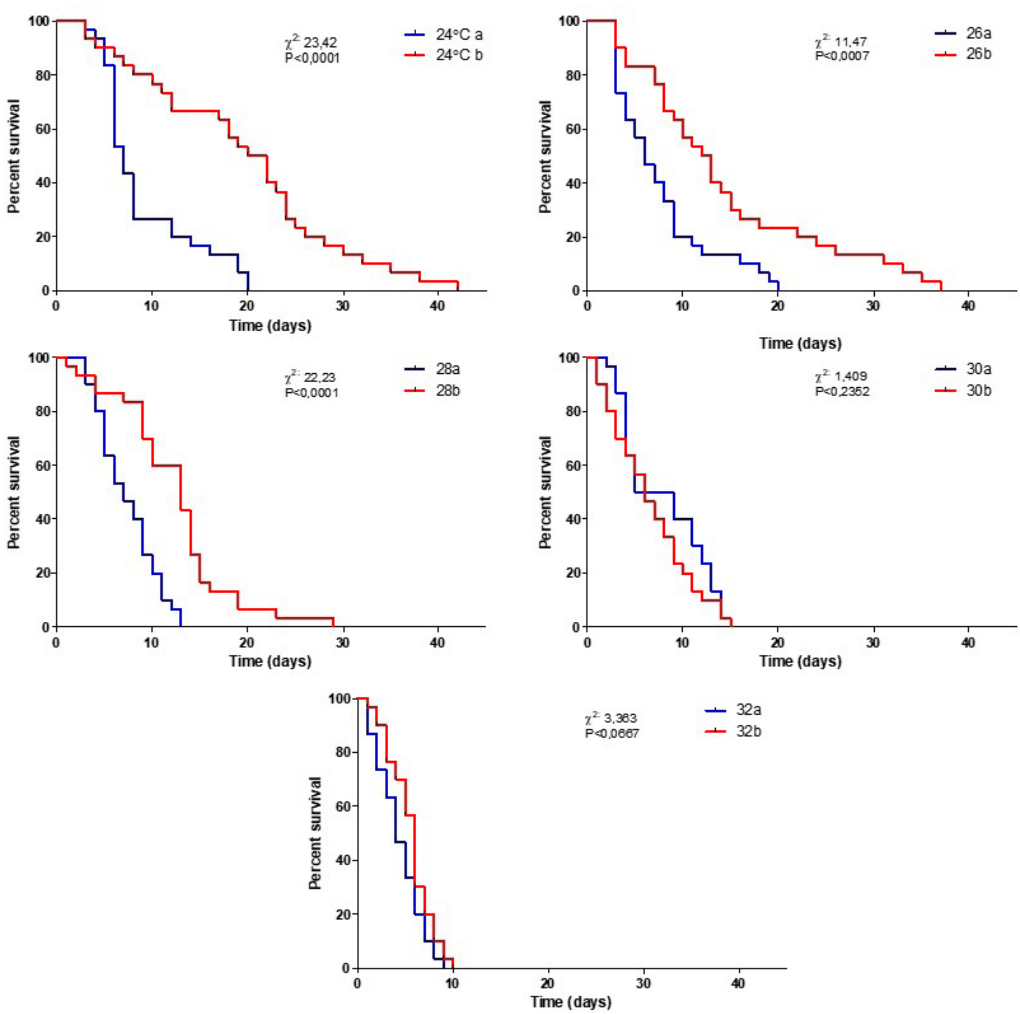


**Figure 5.** Kaplan-Meier estimated survival curves for *Neoechinorhynchus buttnerae* maintained in Eagle's minimum essential medium and with antibiotics at different temperatures, and without (a) or with (b) culture medium renewal.

of time they remained mobile and the culture medium used, were adversely affected, i.e., had gradual swelling that lead immobilization of the parasites.

Minimum essential mediums for culture of parasitic helminths should be composed of a mixture of inorganic and organic salts, and enriched with amino acids, vitamins, glucose and other essential components for cell growth. Different minimum essential medium formulations, such as Dulbecco's modified Eagle's culture medium (DMEM) and Gibco Iscove's modified Dulbecco's culture medium (IMDM) were evaluated for parasitic helminth maintenance and survival time of worms (Yao and Asayama, 2017). In general, good results were achieved in short and long-term *in vitro* culture in viability assays using larvae and adult nematodes (Townson et al., 1986; Njouendou et al., 2017; Heredia et al., 2018; Zofou et al., 2018) and adult trematodes (Uddin et al., 2012). Culture in minimum essential medium has been reported in *in vitro* anthelmintic evaluation against the acanthocephalans *Neoechinorhynchus rutili* and *Echinorhynchus truttae* in rainbow trout (*Oncorhynchus mykiss*) (Taraschewski et al., 1990). Therefore, the use of culture mediums is a fundamental procedure for ensuring that external factors do not influence longevity of helminths during the evaluation of exposure to therapeutic substances or culture assays (Valladão et al., 2020). As can be seen herein, our results prove the *in vitro* efficacy of culture with minimum essential medium for the maintenance of *N. buttnerae* adults, since 100% survival was observed after renewals of minimum essential medium every two days at temperatures between 24 and 28 °C.

Contamination by unidentified substances can occur in the culture mediums and will affect the viability of the parasites (Yao and Asayama, 2017). In the present study, the replacement of the Eagle's minimum essential medium every two days and the supplementation with penicillin and streptomycin also increased the survival time of *N. buttnerae* for up to 42 days when maintained at 24 °C. The antibiotics penicillin and streptomycin are frequently used in the control of bacterial growth (Pawar et al., 2019; Ulkhaq et al., 2020). The combination of penicillin and streptomycin is promising, since penicillin has shown greater efficacy against gram-positive bacteria and streptomycin is more active against aerobic gram-negative bacteria (Madigan et al., 2016). Both antibiotics act by inhibiting bacterial cell wall biosynthesis, initiating protein chain synthesis, or producing defective proteins (Madigan et al., 2016).

The renewal of the different culture mediums is another crucial factor for maintaining the optimal conditions for helminth viability (Costa et al., 2018). However, the effects of renewal or non-renewal of the Eagle's minimum essential medium during the *in vitro* assays with helminths has been poorly adressed. Nicholas and Grigg (1965) reported that during *in vitro* culture of the acanthocephalan *Moliniformis dubius*, even with the use of PBS in the minimum essential medium, these parasites produced acid in 24 h, which was probably due to the release of acidic waste products. The scarcity of nutrients and or the increase in waste products released from living or dead cells are factors that can contribute to the loss of viability of parasites (Sangmaneedet and Smith, 2000).

We hypothesize that the renewal of the minimum essential medium in the present study probably compensated for a significant decrease in pH and maintenance of viable parasites. Previous reports indicate the importance of determining the optimal pH for maintaining the viability of different parasites (Buchmann and Uldal, 1996; Sangmaneedet and Smith, 2000). Therefore, future studies on the final products of the excretory metabolism of *N. buttnerae* may characterize the production of acids by the parasite and determine whether the renewal of the minimum essential medium would therefore be compensating for a significant drop in pH and keeping parasites viable for up to 42 days, as shown herein.

Herein, the renewal or non-renewal of Eagle's minimum essential medium made no difference to the lifespan of *N. buttnerae* when kept at temperatures of 30 and 32 °C, which suggests that it was the elevated temperature that limited the survival of these parasites. The impact of temperature has already been evaluated in a few *in vitro* survival studies of helminths (Hamers et al., 1991; Maya et al., 2010). Costa et al. (2018) observed that temperature had an influence on the morphological integrity of *N. buttnerae* and that, for longer *in vitro* maintenance, the temperature of 24 °C was the most appropriate. In the present study, the viability of *N. buttnerae* in a broader temperature range (from 24 °C to 28 °C) corroborated the results of Costa et al. (2018) who established that a lower temperature is most appropriate for prolonging the survival time of the parasites. Furthermore, dehydration with partial or total deterioration of the body of the parasite at the highest tested temperatures (30 °C and 32 °C) and shorter survival periods were observed. Temperature was also considered a primordial factor in the death of *Spironucleus vortens*, an intestinal parasite of fish, in which the culture was maintained at 34 °C or higher (Sangmaneedet and Smith, 2000).

In a complementary way, it is important to highlight that the seasons influence the abundance of some species of acanthocephalans *in vivo*. For *E. truttae* in *Salmo trutta*, the parasites' life cycle has been related to temperatures of the environment in North America (Awachie, 1965). Gleason (1987) also observed that *Pomphorhynchus bubocolli* exhibited pronounced seasonality in infection rates in North America, which were the highest during winter, which is when the temperatures are lower, and lower during the spring when temperatures are higher. However, in the eastern Amazon, *N. buttnerae* infection in *C. macropomum* occurred only during the dry season when the water temperature is higher, i.e., ranged from 29.0 °C to 31.1 °C (Dias and Tavares-Dias, 2015). It should be noted that the *in vitro* test temperatures of the present study are within the most suitable range for farming *C. macropomum* (i.e. ranged from 25 °C to 27 °C), and had a maximum temperature of 31 °C (Woynárovich and Van Anrooy, 2019).

## 5. Conclusions

The use of Eagle's minimum essential medium with 100 U $mL^{-1}$ of penicillin and 100 µg $mL^{-1}$ of streptomycin, together the renewal of this medium every two days prolonged the survival of *N. buttnerae* when maintained at 24 °C or 28 °C. Therefore, an alternative protocol for the *in vitro* culture of *N. buttnerae* is suggested, and this protocol can be used for further studies aimed at the evaluation of anthelmintics for *in vitro* control of *N. buttnerae*.

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