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DZĪVĪBAS ZINĀTŅU UN TEHNOLOĢIJU INSTITŪTS

ILZE RUBENIŅA

# BENZANTRONA LUMINOFORI TREMATODA UN NEMATODA PARAZĪTU EFEKTĪVAI UN ĀTRAI IZPĒTEI

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## PUBLIKĀCIJU SARAKSTS

Promocijas darbs ir balstīts uz publikācijām, kas disertācijas tekstā ir norādītas ar romiešu cipariem. Oriģinālie raksti ir publicēti ar izdevēju atļaujām.

**I.** Rubenina I., Gavarane I., Kirilova E., Mezaraupe L., Kirjusina M. (2021) Comparison of the Benzanthrone Luminophores: They Are Not Equal for Rapid Examination of *Parafasciolopsis fasciolaemorpha* (Trematoda: Digenea). Biomolecules 11(598):1-15. https://doi.org/10.3390/biom11040598

**II.** Gavārane I., Kirilova E., Rubeniņa I., Mežaraupe L., Osipovs S., Deksne G., Pučkins A., Kokina I., Bulanovs A., Kirjušina M. (2019) A Simple and Rapid Staining Technique for Sex Determination of *Trichinella* Larvae Parasites by Confocal Laser Scanning Microscopy. Microscopy and Microanalysis, 1-7. doi:10.1017/S1431927619015046

**III.** Kirilova E., Kecko S., Mežaraupe L., Gavarāne I., Pučkins A., Mickeviča I., Rubeniņa I., Osipovs S., Bulanovs A., Pupiņš M., Kirjušina M. (2018) Novel luminescent dyes for confocal laser scanning microscopy used in Trematoda parasite diagnostics. Acta Biochimica Polonica 65(3):449-454. https://onlinelibrary.wiley.com/doi/abs/10.1002/bio.3616

**IV.** Gavarane I., Mezaraupe L., Rubenina I., Kirjusina M., Kirilova J. (2018). Staining of economically important parasitic nematodes by developed derivatives of benzanthrone luminophore. 18th International Multidisciplinary Scientific GeoConference SGEM 2018, section Advances in Biotechnology, 581–587. https://doi.org/10.5593/sgem 2018/6.2

Autora ieguldījums (%) pētījumos:

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# IEVADS

## Pētījuma aktualitāte

Mikroskopijai attīstoties, tā būtiski mainīja bioloģijas pētījumu gaitu. Mikroskopu izmanto dažādu dzīvības procesu izpētē, tādējādi raksturojot organismu mikroskopisko uzbūvi. Konfokālā lāzerskenējošā mikroskopija ir viena no metodēm kā pētīt parazītu iekšējo un ārējo struktūru, iegūstot gan detalizētus, gan kvantitatīvus, gan kvalitatīvus datus (Jurberg et al., 2008; Kirjusina et al., 2018; Terenina et al., 2018; Mochalova et al., 2019; Terenina et al., 2020).

Helmintu sugas ir iesaistītas konkurējošās un plēsonīgās mijiedarbībās gan ar saimnieku, gan ar ne-saimnieku taksoniem, tādējādi tiek parādīts parazītu nozīmīgums ekosistēmās un barības ķēdēs (Tompkins et al., 2011). Gan Trematoda klase, gan Nematoda tipa *Trichinella* ģints pārstāvji ir dažādu zoonožu ierosinātāji (Sah et al., 2020). Trematoda klase ietver šistosomu un pārtikas bojājošu trematodes pārstāvjus, kuri invadējuši vairāk kā 294 miljonu cilvēku visā pasaulē(*World Health Organisation*, 2015, 2019; Bennett & Robinson, 2021). Savukārt, *Trichinella* ģints parazīta izraisītā zoonoze – trihineloze ir ierindota pasaules cilvēkam bīstamāko slimību topa desmitniekā (Pozio & Zarlenga, 2019). Pēc Pasaules Veselības Organizācijas datiem, ik gadu 10 000 cilvēku tiek reģistrēta invadēšanās ar trihinellu (*World Health Organization*, 2022).

Izmantojot fluorescento mikroskopiju, liela nozīme tiek piešķirta luminoforiem (Ndao, 2009). Savulaik parazītu struktūras krāsošanā tika plaši izmantotas fluorescējošās krāsvielas akridīna oranžais un rodamīns C (Geller & Timonov, 1969a,b; Stankiewicz et al., 1996; Janssen 1998), bet mūsdienāssāka parādīties citas sintezētās krāsvielas bioloģisko organismu morfoloģiskai izpētei (Dapson, 2007; Fakhar & Ghobaditara, 2016). Pēdējo gadu laikā popularitāti guvuši benzantrona luminofori, to izmantošana dod iespēju noteikt specifiskus lipīdus un proteīnus (Zhytniakivska et al., 2014a; Ryzhova et al., 2016). Benzantrona luminofori ir zināmas kā fluorescentās zondes, kas atkarībā no krāsas struktūras emitē noteiktā, spektrālā reģionā: dzeltens – zaļš vai sarkans - violets (Khrolova et al., 1984). Daži benzantronu luminofori tiek izmantoti membrānu strukturālo izmaiņu novērošanai un nukleīnskābju savienošanās izpētei (Dobretsov, 1989; Yang et al., 1999). Pētījumi apstiprinājuši, ka luminoforus var izmantot bioloģisku objektu krāsošanai. Lai gan krāsošana ar benzantrona luminoforiem krietni vien samazina paraugu sagatavošanas laiku, katrai organismu grupai ir jāpiemeklē specifisks benzantrona luminofors (Kirjusina et al., 2018; Kirilova et al., 2019).

Pētījumos aprakstītie trematodes paraugu sagatavošanas standarta protokoli konfokālai lāzerskenējošai mikroskopijai aizņem vairāk kā vienu vai pat divas dienas (Krupenko, 2014; Krupenko & Dobrovolskij; 2018; Krupenko, 2019) un ir koncentrēti uz kādu noteiktu sistēmu izpēti, piemēram, muskuļu sistēmu

(Krupenko, 2019), nervu sistēmu (Kremnev et al., 2020, 2021). Paraugu sagatavošanas posmā tiek izmantoti dažādi paraugu mazgāšanas un fiksēšanas šķīdumi, piemēram, paraformaldehīds, fosfātu buferšķīdums jeb PBS ar 0.1% nātrija azīdu, u.c. (Krupenko & Gonchar, 2017a,b; Krupenko et al., 2016). Rezultātā izstrādātie protokoli prasa daudz resursu un cilvēka darba stundu. Savukārt, trihinellas krāsošanas protokoli bieži vien nav paredzēti paša kāpura struktūras noteikšanai, bet gan specifisku procesu novērošanai, piemēram, imūnsistēmas atbildes reakcijai (Bai et al., 2012), anti-trihinellu vakcīnu pārbaudei (Hu et al., 2021), noteiktu proteīnu lokalizēšanai (Hernandez-Bello et al., 2008; Morales-Montor, 2022), mijiedarbības izpētei starp nematodi un muskuļu šūnām *in vitro* (Bai et al., 2011). Tomēr trūkst luminoforu, kas spētu iekrāsot parazītu vispārējo struktūru, nepatērējot daudz resursu un laika parauga sagatavošanai konfokālai lāzerskenējošai mikroskopijai.

Parazītu mikroskopiskā izpēte ietver dzimumu noteikšanu. Viens no populāciju raksturlielumiem ir dzimumstruktūra. Kozek (1975) un Li et al. (1999) ir mēģinājuši noteikt trihinellu kāpuru dzimumus, uzskaitot morfoloģiskās pazīmes, kuras atšķir mātīti no tēviņa. Tomēr, mēģinājumi nav bijuši veiksmīgi, jo, izmeklējot paraugus ar gaismas mikroskopiju, ir samērā grūti atšķirt pazīmes, kuras prasa lielu precizitāti (Weller, 1943; Kozek, 1975). Viena no pazīmēm pēc kuras ir iespējams noteikt kāpuru dzimumu ir gala zarnas garums, bet ar gaismas mikroskopiju iegūtie mērījumi var būt neprecīzi (Villella, 1966).

**Pētījuma novitāte**

Trematodes parazītu sugu pētījumos aprakstītie helmintu izpētes protokoli, izmantojot konfokālo lāzerskenējošo mikroskopiju, lielākoties ir ražotāju izstrādāti standarta protokoli ar nelielām modifikācijām. Paraugu sagatavošana aizņem vienu vai pat divas dienas (Krupenko, 2014; Krupenko & Dobrovolskij; 2018; Krupenko, 2019), turklāt krāsošanas protokoli ir vērsti uz konkrētu orgānu sistēmu izpēti (Krupenko, 2019; Kremnev et al., 2020, 2021). Savukārt, trihinellas krāsošanas protokoli tiek vērsti uz specifisku procesu novērošanu vai noteiktu proteīnu lokalizēšanu (Hernandez-Bello et al., 2008; Bai et al., 2011, 2012; Hu et al., 2021; Morales-Montor, 2022). Tomēr trūkst luminoforu un krāsošanas protokolu, kas koncentrētos uz paša parazīta vispārējās struktūras izpēti, nepatērējot daudz laboratorijas resursu un laiku parauga sagatavošanai konfokālai lāzerskenējošai mikroskopijai.

Dotā pētījuma gaitā tika sintezēti septiņi benzantrona luminofori (AM323, AZPP, AM1, AM2, AM4, AM16, P8,) un izstrādāti noteikti krāsošanas protokoli Trematoda klases dažādu parazītu sugu izpētei, izmantojot konfokālo lāzerskenējošo mikroskopiju. Izstrādātie krāsošanas protokoli ir piemēroti efektīvai un ātrai dažādos ķīmiskos fiksatoros fiksētiem *P. fasciolaemorpha* (ad.), *D. spathaceum* (ad.), *D. subclaviatus* (mtc.) un *P. confusus* (ad.) izpētei. Tika sintezēti divi benzantrona luminofori (AZP un P13) un izstrādāti noteikti krāsošanas protokoli Nematoda tipa parazītu sugu izpētei. Izstrādātie

krāsošanas protokoli ir piemēroti trīs dažādu trihinellu paraugu tipu izpētei: paraugiem, kuri uzglabāti 96,6% etanolā; kāpuriem, kas sasaldēti dzīvnieku muskulatūrā vai nesen ievākta dzīvnieku muskulatūra, no kuras vienas dienas laikā izdalīti trihinellas kāpuri un tie fiksēti. Rezultātā tika novērotas morfoloģiskas atšķirības starp *T. spiralis* un *T. britovi* sugu paraugiem. Pētījuma laikā tika sintezēts AZM luminofors un izstrādāts noteikts krāsošanas protokols *T. spiralis* un *T. britovi* kāpuru dzimumu noteikšanai, balstoties uz kāpura gala zarnas garumu.
Piemeklējot specifisku benzantrona luminoforu dažādām parazītu sugām, tika paaugstināta izstrādāto protokolu efektivitāte. Izstrādātie krāsošanas protokoli ietaupa paraugu sagatavošanas laiku un laboratorijas resursus, jo paraugu sagatavošanai konfokālai lāzerskenējošai mikroskopijai nav jāvelta vairākas stundas vai dienas.

**Darba hipotēze**
Dažādu parazītu sugu efektīvai un ātrai izpētei ar konfokālo lāzerskenējošo mikroskopiju ir piemērots noteikts krāsošanas protokols, izmantojot noteiktu ķīmisko fiksatoru un specifisku benzantrona luminoforu.

**Darba mērķis**
Izstrādāt krāsošanas protokolu ar benzantrona luminoforiem Trematoda klases un Nematoda tipa dažādu parazītu sugu efektīvai un ātrai izpētei, izmantojot konfokālo lāzerskenējošo mikroskopiju.

**Darba uzdevumi**
1. Aprobēt sintezētos benzantrona luminoforus Trematoda klases un Nematoda tipa dažādu parazītu sugu efektīvai un ātrai izpētei (**I, II, III, IV**).
2. Izstrādāt krāsošanas protokolu, izmantojot specifisku benzantrona luminoforu, Trematoda parazītu sugu paraugiem, fiksētiem dažādos ķīmiskos fiksatoros, efektīvai un ātrai izpētei (**I, III**).
3. Izstrādāt krāsošanas protokolu, izmantojot specifisku benzantrona luminoforu, trīs dažāda tipa Nematoda parazītu sugu paraugiem, fiksētiem dažādos ķīmiskos fiksatoros, efektīvai un ātrai izpētei un dzimuma noteikšanai (**II, IV**).
4. Izanalizēt sintezētā benzantrona luminofora piemērotību un krāsošanas protokola efektivitāti Trematoda klases un Nematoda tipa parazītu sugu ātrai izpētei, pielietojot konfokālo lāzerskenējošo mikroskopiju (**I, II, III, IV**).

**Aizstāvāmās tēzes**
1. Sintezētie benzantrona luminofori ir piemēroti Trematoda klases un Nematoda tipa dažādu parazītu sugu efektīvai un ātrai izpētei, izmantojot konfokālo lāzerskenējošo mikroskopiju.
2. Ķīmiskie fiksatori ietekmē pētāmā parauga detalizētu izpēti un noteiktam luminoforam ir piemērots noteikts fiksators.

3. Trematodes parazītu krāsošanas soļa ilgumu ietekmē pētāmā parauga izcelsme – poikiloterms saimnieks vai homoterms saimnieks.
4. Efektīva un ātra protokola izstrāde sniedz detalizētus rezultātus divu stundu laikā.
5. Specifisks benzantrona luminofors un noteikts krāsošanas protokols ir piemērots *T. spiralis* un *T. britovi* kāpuru dzimuma noteikšanai.

**Pētījuma rezultātu aprobācija**

*Zinātniskā monogrāfija:*

Visu autoru ieguldījums monogrāfijas tapšanā ir vienāds, neatkarīgs no autoru pieminēšanas secības.

Gavarane I., Kirilova E., Rubenina I., Osipovs S., Mezaraupe L., Puckins A., Kirjusina M. 2020. Simple and rapid luminiscent staining protocols in Helmintology. [Monograph]. Published by Daugavpils University "Saule", Daugavpils, 131 pages.

*Patents*:

Gavarāne I., Rubeniņa I., Kirilova J., Kirjušina M. Luminiscenta metode *Trichinella* spp. parazīta kāpura dzimuma noteikšanai. Patenta pieteikuma publikācija. Latvijas Republikas Patentu valdes oficiālais izdevums. Izgudrojumi, preču zīmes un dizainparaugi 2020-03. Latvijas Republikas Patentu valde, 2020. ISSN 2255-9655. Patents iesniegts 20.12.2019. (Pieteikuma nr. P-19-76). Patenta publikācijas datums 20.03.2021., Patenta reģistra nr. 15489.

Par promocijas darba rezultātiem tika ziņots *starptautiskās zinātniskās konferencēs:*

1. Fridmans R., Kirilova J., Mežaraupe L., Rubeniņa I., Kirjušina M. 2021. Synthesis of new benzanthrone derivatives with imine and amine groups. Abstracts of the 63 Scientific conference of Daugavpils University, Daugavpils, Latvia, 15-16 April, Abstract Book, p. 33 *(mutisks ziņojums*)
2. Bordjuga V., Mežaraupe L., Rubeniņa I., Kirjušina M. 2021. Development of staining protocol using benzanthrone luminophores for trematodes muscle research. Abstracts of the 63 Scientific conference of Daugavpils University, Daugavpils, Latvia, 15-16 April, Abstract Book, p. 30 (*mutisks ziņojums*)
3. Širokova J., Mežaraupe L., Rubeniņa I., Kirjušina M. 2021. Comparison of different fluorescent dyes for trematoda *Prototocus confusus* imaging. Abstracts of the 63 Scientific conference of Daugavpils University, Daugavpils, Latvia, 15-16 April, Abstract Book, p. 28 (*mutisks ziņojums*)
4. Rubenina I., Gavarane I., Mezaraupe L., Gravele E., Pupins M., Kirjusina M. 2021. Description of *Prosotocus confusus* in *Phelophylax esculentus* complex and *Rana temporaria* from Latvia. Abstract book, 79. scientific conference Zoology and Animal Ecology, Univerity of Latvia, Riga, Latvia, January 28, 2021, Abstract Book p. 26 (*mutisks ziņojums*)

5. Kirjušina M., Gavarāne I., Mežaraupe L., Pupiņš M., Kirilova E., Rubeniņa I. 2019. Luminophore AM1 for examination of adult *Prosotocus confusus* by confocal laser scanning microscopy. October 09-11, 2019. Copenhagen, Denmark, the 8th Conference of the Scandinavian-Baltic Society for Parasitology (SBSP) and the Annual Meeting of the European Veterinary Parasitology College (EVPC), Abstract Book, p. 72 (*stenda ziņojums*)
6. Gavarāne I., Rubeniņa I., Mežaraupe L., Kirilova E., Kirjušina M. 2019. Luminophores for *Trichinella britovi* larvae examination and sex determination using confocal laser scanning microscopy. October 09-11, 2019. Copenhagen, Denmark, the 8th Conference of the Scandinavian- Baltic Society for Parasitology (SBSP) and the Annual Meeting of the European Veterinary Parasitology College (EVPC), Abstract Book, p. 64 (*stenda ziņojums*)
7. Rubeniņa I., Mežaraupe L., Gavarāne I., Kirilova E., Kirjušina M. 2019. Perspectives in studies of adult *Parafasciolopsis fasciolaemorpha* by confocal laser scanning microscopy. October 09-11, 2019. Copenhagen, Denmark, the 8th Conference of the Scandinavian-Baltic Society for Parasitology (SBSP) and the Annual Meeting of the European Veterinary Parasitology College (EVPC), Abstract Book, p. 30 (*mutisks ziņojums*)
8. Rubeniņa I., Kirilova E., Gavarāne I., Mežaraupe L., Kirjušina M. 2019. Benzanthrone luminophores are not equal for simple rapid examination of liver parasite *Parafasciolopsis fasciolaemorpha* (Trematoda: Digenea). September 25th - 28th, 2019. Poznan, Poland, International Conference on Biotechnology and Bioengineering, Abstract Book p. 25 (*stenda ziņojums*)
9. Gavarāne I., Rubeniņa I., Kirilova E., Kirjušina M. 2019. Study of fish parasites *Dactylogyrus* by confocal laser scanning microscopy. 3-6 October 2019. Jahorina, Bosnia and Herzegovina, X International Agriculture Symposium "AGROSYM 2019", Book of Abstracts, 716 pages (*stenda ziņojums*)
10. Kirjušina M., Rubeniņa I., Kirilova E., Gavarāne I. 2019. Benzanthrone luminophores for examination of fish *Diplostomum* metacercariae. 3-6 October 2019. Jahorina, Bosnia and Herzegovina, X International Agriculture Symposium "AGROSYM 2019", Book of Abstracts, 719 pages (*stenda ziņojums*)
11. Gavarāne I., Rubeniņa I., Mežaraupe L., Bulanovs A., Kirjušina M., Kirilova J. 2019. Novel benzanthrone luminophore AZM for *Trichinella britovi* larvae sex determination. 17-19 June 2019. Riga, Latvia, FEBS3+ Conference (*stenda ziņojums*)
12. Kirjušina M., Gavarāne I., Mežaraupe L., Rubeniņa I., Kecko S., Kirilova E. 2019. Application of novel synthesized luminophores for staining of adult Trematoda in confocal laser scanning microscopy. May 12-18, 2019, Oludeniz, Turkey, INTERIM 2019 6th International congress on microscopy & spectroscopy (*stenda ziņojums*)

13. Mežaraupe L., Kecko S., Kirilova E., Gavarāne I., Kirjušina M., Rubeniņa I. 2019. Staining Of Trematode *Parafasciolopsis Fasciolaemorpha* (Fasciolidae) With Luminiscent Benzanthrone AM323. 02-04 May 2019. Kaunas, Lithuania, 3rd International Conference „Smart Bio", Book of Abstracts, 213 pages (*stenda ziņojums*)
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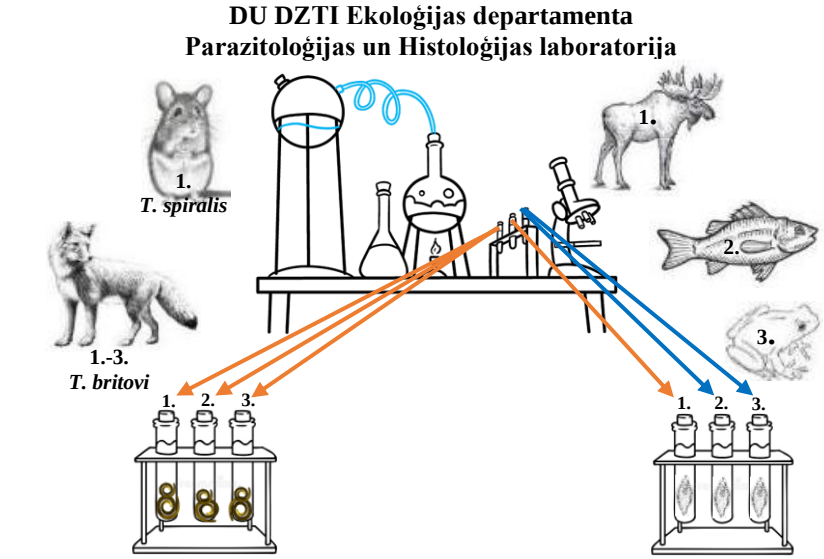
# MATERIĀLS UN METODES

## Bezantrona luminoforu sintēzes apraksts (I, II, III, IV)

Daugavpils Universitātes (DU) Dzīvības Zinātņu un Tehnoloģiju Institūta (DZTI) Lietišķās ķīmijas departamentā tika sintezēti deviņi benzantrona luminofori: AZPP, AM323, AZM, AM1, AM2, AM4, AM16, P8 un P13, kuri pētījuma gaitā tika izmantoti bioloģisko objektu krāsošanā un specifisku krāsošanas protokolu izstrādē, efektīvai un ātrai dažādu Trematoda klases un Nematoda tipa parazītu sugu izpētei.

## Pētāmais materiāls (I, II, III, IV)

Pētījuma gaitā tika izmantoti helmintu paraugi no DU DZTI Ekoloģijas departamenta Parazitoloģijas un Histoloģijas laboratorijas. Helminti tika iegūti no dažādiem saimniekiem: peles (**II**), rudās lapsas (**II, IV**), aļņiem (**I**), zivīm (**III**) un bezastainiem abiniekiem (**III**). Izmantoto materiālu raksturojums atspoguļots 1. attēlā.




**1. attēls.** Pētījumā izmantotais materiāls (Rubeniņa, 2022)

**Pētāmā materiāla ķīmiskā fiksēšana (I, II, III, IV)**

Tūlīt pēc izdalīšanas no saimnieka organisma Trematoda parazītu sugu paraugi tika ķīmiski fiksēti sešos dažādos fiksatoros (skat. 1. tabulu).

1. tabula

**Trematoda parazītu sugu paraugu ķīmiskā fiksēšana un uzglabāšana**

| Ķīmiskais fiksators | 70% etanols **(I)** | 96 % etanols **(I, III)** | AFA šķīdums **(I, III)** | Karnoja šķīdums **(I)** | Bouina šķīdums **(I)** | 10% neitrāli buferēts formalīns **(I)** |
|---|---|---|---|---|---|---|
| Fiksēšanas ilgums *P. fasciolaemorpha* | Līdz izmeklēšanai | Līdz izmeklēšanai | 2 h | 2 h | 2 h | Līdz izmeklēšanai |
| Fiksēšanas ilgums saldūdens trematodēm | - | Līdz izmeklēšanai | 1 h | 1 h | - | - |
| Skalošana *P. fasciolaemorpha* | Nav piemērojama | Nav piemērojama | 70% etanols | 70% etanols | 70% etanols | Nav piemērojama |
| Skalošana saldūdens trematodēm | - | Nav piemērojama | 96 % etanols | 96 % etanols | - | - |
| Ķīmiskais fiksators, kurā tika uzglabāti *P. fasciolaemorpha* | 70% etanols | 96 % etanols | 70% etanols | 70% etanols | 70% etanols | 10% neitrāli buferēts formalīns |
| Ķīmiskais fiksators, kurā tiek uzglabātas saldūdens trematodes | - | 96 % etanols | 96 % etanols | 96 % etanols | - | - |
| Uzglabāšana | ledusskapī +4°C temperatūrā līdz nākamai izmeklēšanai | | | | | |

- ķīmiskais fiksators netika izmantots materiāla fiksēšanai

Izolētie *T. spiralis* un *T. britovi* kāpuru paraugi uzreiz pēc izdalīšanas no dzīvnieku muskulatūras 1 stundu tika fiksēti četros dažādos ķīmiskos fiksatoros (skat. 2. tabulu).

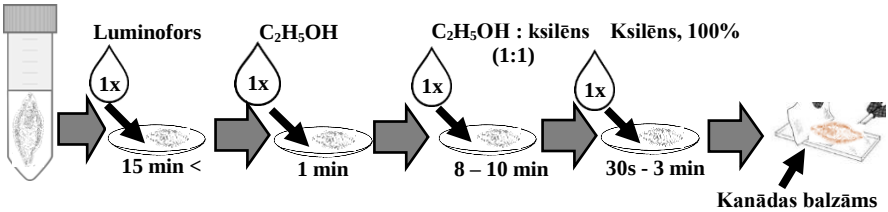
2. tabula

***T. spiralis* un *T. britovi* parazītu sugu paraugu ķīmiskā fiksēšana un uzglabāšana**

| Ķīmiskais fiksators | 70% etanols (**II**) | 96,6 % etanols (**IV**) | AFA šķīdums (**II**) | Karnoja šķīdums (**II**) | Bouina šķīdums (**II**) |
|---|---|---|---|---|---|
| Fiksēšanas ilgums | Līdz izmeklēšanai | Līdz izmeklēšanai | 1 h | 1 h | 1 h |
| Skalošana | Nav piemērojama | Nav piemērojama | 70% etanols | 70% etanols | 70% etanols |
| Ķīmiskais fiksators, kurā tika uzglabāti kāpuri | 70% etanols | 96,6% etanols | 70% etanols | 70% etanols | 70% etanols |
| Uzglabāšanas temperatūra | ledusskapī +4°C līdz nākamajai izmeklēšanai | ledusskapī +4°C līdz nākamajai izmeklēšanai | ledusskapī +4°C līdz nākamajai izmeklēšanai | ledusskapī +4°C līdz nākamajai izmeklēšanai | ledusskapī +4°C līdz nākamajai izmeklēšanai |
| Uzglabāšanas ilgums | Diena līdz 1 gadam | 1 – 5 gadi | Diena līdz 1 gadam | Diena līdz 1 gadam | Diena līdz 1 gadam |

**Krāsošanas protokoli ar specifisku benzantrona luminoforu (I, II, III, IV)**

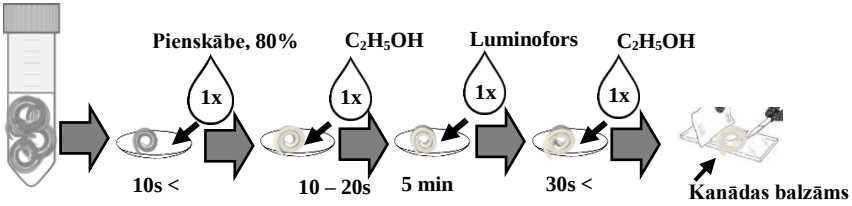
Benzantrona luminofori AZPP un AM323 tika sintezēti *P. fasciolaemorpha* paraugiem, kuri fiksēti 70% un 96% etanolā, AFA, Karnoja un Bouina šķīdumā vai 10% neitrālā formalīna buferšķīdumā (**I**). Parazīts tika izdalīts no aļņa (*Alces alces*) (homoterma saimnieka). Trematoda paraugu vispārēja krāsošanas gaita ir atspoguļota 2. attēlā.




**2. attēls.** Vispārēja krāsošanas gaita trematodes paraugiem (Rubeniņa, 2022)

Benzantrona luminofori AM1, AM2, AM4, AM16 un P8 tika sintezēti saldūdens trematodēm: *D. spathaceum* (mtc.), kuras fiksētas 96% etanolā; *D. subclaviatus* (ad.), kuras fiksētas Karnoja šķīdumā un *P. confusus* (ad.), kuri fiksēti AFA šķīdumā (**III**). Izstrādātais krāsošanas protokols tika pielietos paraugiem, kuri iegūti no poikilotermiem saimniekiem: bezastainiem abiniekiem (*D. subclaviatus*, *P. confusus*) un saldūdens zivīm (*D. spathaceum*).

Benzantrona luminofors AZM tika sintezēts kāpuriem, kuri izolēti no dzīvnieku muskulatūras un fiksēti 70% etanolā, Karnoja, Bouina vai AFA šķīdumā. Savukārt, luminofors P13 tika sintezēts *T. britovi* kāpuriem, no kuriem daļa kāpuru bija fiksēta 96,6% etanolā no 1 līdz 5 gadiem un otra daļa kāpuru bija sasaldētā dzīvnieku muskulatūrā no 1 līdz 5 gadiem. Nematoda paraugu vispārēja krāsošanas gaita ir atspoguļota 3. attēlā.




**3. attēls.** Vispārēja krāsošanas gaita nematodes paraugiem (Rubeniņa, 2022)

Izstrādātais trihinellu kāpuru krāsošanas protokols, izmantojot luminoforu AZM, tika pielietots trihinella kāpura dzimuma noteikšanai. Dzimuma noteikšana tika balstīta uz Kozek (1975) un Liu et al. (1991) pētījumiem, kuros tika noskaidrots, ka tēviņa gala zarnas garums var sasniegt 63 μm, bet mātītēm tas vienmēr ir uz pusi mazāks – 30 μm.

**Konfokālā lāzerskenējošā mikroskopijas metode (I, II, III, IV)**

Visi parazīti pēc krāsošanas ar benzantrona luminoforu tika pētīti, izmantojot *Nikon Eclipse Ti-E* konfokālo lāzerskenējošo mikroskopu (Nikon, Japāna), kas ir konfigurēts ar ātrgaitas multifotonu *A1R MP* konfokālo sistēmu un aprīkots ar digitālo DS-U3 kameru (Nikon, Japāna). Fluorescences ierosināšanai tika izmantoti trīs lāzeri: *Ch2* zaļais lāzeris λ= 488 nm ar FITC filtru (500–550 nm) (**I, II, III, IV**)); *Ch3* oranžais lāzeris λ= 561 nm ar TRICT filtru (570-620 nm) (**III, IV**) un *Ch4* sarkanais lāzeris λ= 638 nm ar Cy5 filtru (662-737 nm) (**I**). Visi iegūtie dati tika saglabāti .jpg un *ND2 Image File* formātā, pēc tam dati tika apstrādi ar *NIS Elements Advanced Research 3.2 64-bit* programmatūru (Nikon, Japāna).

# REZULTĀTI

## Trematoda un Nematoda paraugu fiksēšana un uzglabāšana (I, II, III, IV)

Ķīmisko fiksatoru iedarbība uz paraugiem tika novērota divas reizes: laikā, kad paraugs tika sagatavots uzglabāšanai un konfokālās lāzerskenējošās mikroskopijas (KLSM) laikā.

*P. fasciolaemorpha* (**I**) ķīmiskā fiksēšana tika veikta sešos dažādos fiksatoros. Fiksēšanas laikā tika novērots vai paraugos notiek kādas morfoloģiskas izmaiņas. Trematodes fiksēšana Karnoja šķīdumā izraisīja paraugu izmēru palielināšanos. Etanolā fiksētie paraugi kļuva nedaudz tumšāki, salīdzinot ar paraugu krāsu pirms fiksēšanas. Bouina šķīduma fiksators visus paraugus krāsoja dzeltenā krāsā un tālākā paraugu apstrādē to nebija iespējams izskalot. Rezultātā Bouina šķīdumā fiksētie *P. fasciolaemorpha* ietekmēja iegūto rezultātu kvalitāti izpētē ar KLSM.

Saldūdens trematodes (**III**) tika uzglabātas 96% etanolā, ledusskapī +4°C temperatūrā līdz nākamai izmeklēšanai. Nedēļu glabātos *D. spathaceum* (mtc.) un *D. subclaviatus* (ad.) netika novērotas fiziskas izmaiņas. *P. confusus* paraugos tika novērotas morfoloģiskas izmaiņas, jo krāsošanas laikā paraugs sadalījās, tas bija kļuvis pārāk trausls. KLSM laikā paraugos tika novēroti fiziski pārrāvumi ķermenī – melni plankumi.

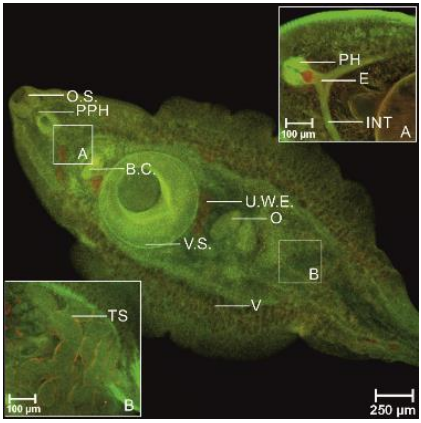
Pētījuma laikā tika izmantoti no dzīvnieku muskulatūras izolētie trihinellas kāpuri (**II**), kas tika glabāti 96,6% etanolā vai kāpuri, kas tika iegūti no –20°C temperatūrā sasaldētas muskulatūras (**IV**). Tūlīt pēc izolēšanas no dzīvnieku muskulatūras, trihinellas kāpuri tika fiksēti piecos dažādos ķīmiskos fiksatoros. Pētot paraugus ar gaismas mikroskopiju, paraugos netika novēroti fiziski bojājumi. Savukārt, izmeklējot paraugus ar KLSM, atklājās, ka vecākos paraugos (3 gadi <) ir novērojama morfoloģiskās struktūras degradācija.

Pētījumi apstiprināja, ka ķīmiskajam fiksatoram ir ietekme detalizētu KLSM datu ieguvē.

## Trematoda un Nematoda paraugu efektīva un ātra izpēte (I, II, III, IV)

Pirms paraugu fiksēšanas un krāsošanas procedūrām, tika veikta kontroles parauga izpēte. Autofluorescences pētījumi tika veikti ar paraugiem, kuri vienas dienas laikā tika izolēti no saimnieka vai sasaldētas saimnieka muskulatūras. Izolētie paraugi netika ķīmiski fiksēti un netika krāsoti ar benzantrona luminoforiem (**I, III**). Nefiksētie un nekrāsotie paraugi tika izpētīti ar KLSM metodi. Autofluorescences rezultātā trematodes paraugiem tika izgaismota vispārēja ķermeņa forma, kur tika saskatīts, ka ķermeņa virsma nav gluda. *P. fasciolaemorpha* paraugos bija iespējams noteikt vēdera piesūcekņa un kopulācijas orgāna novietojumu. *T. spiralis* un *T. britovi* autofluorescences eksperimentos tika izmantoti ķīmiskos fiksatoros fiksēti paraugi (**II**). Rezultāti apstiprināja, ka, izmantojot 488 nm lāzeri, ir iespējams sasniegt gandrīz 10 reizes autofluorescences vājināšanas signālu salīdzinājumā ar 405 nm viļņu garuma absorbciju (**II, IV**).

*P. fasciolaemorpha* parazītu krāsošana tika veikta paralēli ar abām krāsām, iegūto rezultātu salīdzināšanai. Trematodes ķermeņa izpēte (ar AZPP krāsotiem paraugiem) bija iespējama jau zem x40 palielinājuma (skat. 4. attēlu).

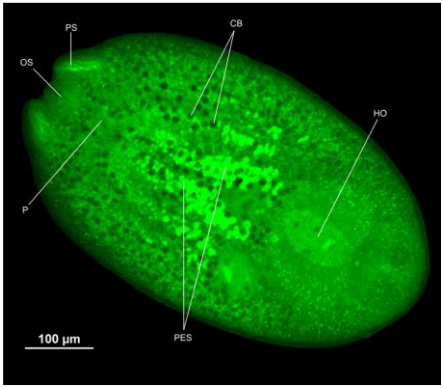



**4. attēls.** *Parafasciolopsis faciolaemorpha* (ad.) krāsota ar AZPP, fiksators AFA (viens optiskais griezums) (Rubeniņa, 2021). O.S.— mutes piesūceknis, PPH — pre-rīkle, PH — rīkle, E — barības vads, C — kopulācijas orgāns, V — dzeltenuma dziedzeris, TS — sēklinieki, O — olnīcas, INT — zarnas, V.S. — mutes piesūceknis, U.W.E. — dzemde pildīta ar olām

Tika novērota ķermeņa virsmas struktūras, tajā skaitā ķermeņa virsmas dzelkšņi un teguments. Tika vizualizēta ķermeņa telpiskā (dimensiju) struktūra. Zem x100 palielinājuma vienlaicīgi tika novēroti trīs muskuļu slāņi: cirkulārais, diagonālais un gareniskais. Mutes un vēdera piesūcekņos tika novērotas cirkulārais, gareniskais un radiālais muskuļu slānis. Trematodes ķermeņa astes zonā tika skaidri novērotas parenhīmas šūnas. Rezultāti apstiprināja, ka vispiemērotākā luminofora un fiksatora savienība *P. fasciolaemorpha* izpētei ar KLSM ir 70% etanols vai AFA šķīdums un AZPP luminofors. Izstrādātais krāsošanas protokols, izmantojot sintezēto AZPP benzantrona luminoforu un KLSM, ir piemērots *P. fasciolaemorpha* efektīvai un ātrai izpētei, padarot krāsošanas protokolu mazāk darb un laikietilpīgu (**I**).

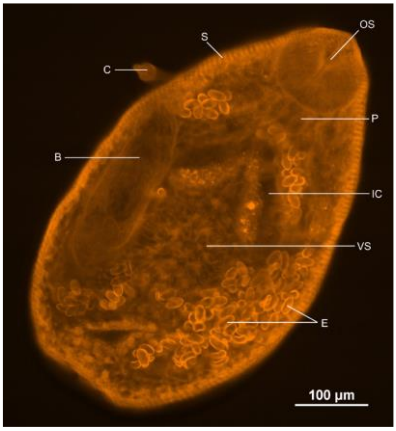
*D. spathaceum* mtc., fiksēti 96% etanolā, mikroskopiskās izpētes rezultāti norādīja uz AM1, AM2, AM4, AM16 un P8 luminoforu piemērotību trematodes izpētei. Spilgta luminicence tika novērota skoleksā, pseido piesūcekņos un ekskretorā sistēmā (**III**).

KLSM rezultāti pētījumos ar *D. subclaviatus* trematodēm, fiksētas Karnoja šķīdumā, uzrādīja detalizētākus datus, iezīmējot sīkus, taksonomiski nozīmīgus skeleta elementus (skat. 5. attēlu), bet no otras puses, paraugos nebija novērojami iekšējie orgāni un struktūras, pat mainot skenēšanas iestatījumus (**III**).




**5. attēls.** *Diplostomum spathaceum* (mtc.) krāsots ar AM2 (Rubeniņa, 2018). OS – mutes piesūceknis; PS – pseido piesūceknis; P – rīkle; HO – skoleks; CB – kaļķu ķemenīši; PES – primārā ekskretorā sistēma.

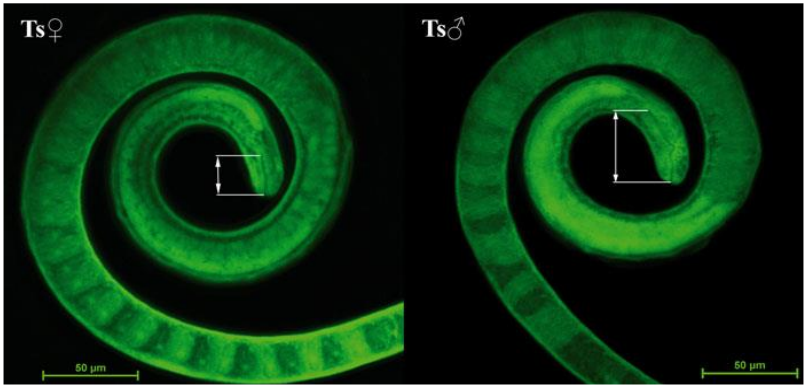
*P. confusus* paraugos tika novērots, ka kutikulu klāj dzelksnīši. Tika novērota gremošanas un reproduktīvā sistēma (skat. 6. attēlu) (**III**).




**6. attēls.** Pieaudzis *Prosotocus confusus* krāsots ar AM1 (iekšējā struktūra) (Rubeniņa, 2018)
OS – mutes piesūceknis, P – rīkle, IC – zarnu zars; VS – vēdera piesūceknis; E – oliņas; S – dzelkšņi; B – cirrus maiss jeb bursa; C – kopulācijas orgāns jeb cirrus.

*T. spiralis* un *T. britovi* kāpuru izpētei tika sintezēti P13 un AZM benzantrona luminofori. Benzantronu luminoforu un KLSM metodes izmantošana deva iespēju detalizēti izpētīt parazīta ķermeni, ieskaitot orgānu morfoloģiju, to

izkārtojumu ķermenī. Sintezēto luminoforu un izstrādāto krāsošanas protokolu efektivitāte tika pārbaudīta, aizstājot luminoforu ar komerciāli pieejamu krāsvielu. Rezultāti apstiprināja kvalitatīvāku datu ieguvi ar sintezētajiem P13 un AZM benzantroniem, pateicoties kuriem tika novērotas atšķirības kutikulas struktūrā starp abām trihinellu sugām. *T. spiralis* kutikulā tika novērota "pseidosegmentācija", bet *T. britovi* kutikulā segmentācija netika novērota, kas lika secināt, ka tā ir gluda. Ar AZM izstrādātais protokols deva iespēju precīzi izmērīt kāpuru gala zarnas garumus, balstoties uz zarnas garumu ir iespējams noteikt kāpura dzimumu (skat. 7. attēlu). Rezultātā tika aprēķināts, ka vidējais gala zarnas garums *T. britovi* mātītēm bija 21,19 µm ±2,45 SD un *T. spiralis* - 20.55 µm ± 1.48 SD. Tēviņiem vidējais gala zarnas garums bija 41,08 µm ±4,26 SD un 46.08 µm ± 2.95 SD. Vēlāk tika novērota spilgta fluorescence mātītes dzemdes aizmetnī.




**7. attēls**. KLSM attēlā atzīmēta gala zarna gan trihinella mātītei, gan tēviņam (Rubeniņa, 2019). Ts♀—*T. spiralis* mātīte, Ts♂—*T. spiralis* tēviņš. Ar ↔ atzīmēta gala zarna.

# DISKUSIJA

Mūsdienās ar vien lielāka uzmanība tiek pievērsta bioloģisko objektu krāsošanai ar luminoforiem (Shivraj et al., 2018). Iepriekšējos pētījumos tika apstiprināts, ka benzantrons (3-N-2-[4-(2 feniletil)piperazin-1-il]acetamidobenzantrons (AZP5) ir piemērots helmintu, glabāti 96% etanolā, morfoloģiskai izpētei (Kirjusina et al., 2018). Kirilova et al. (2019) apstiprināja benzantronu luminofora izmantošanu kallusa embrija šūnu vizualizēšanai. Šajā kontekstā tika sintezēti jauni benzantronu luminofori, kas vizualizētu šūnas membrānu, krāsai lokalizējoties membrānas lipīdos (**I, II, III, IV**). Piemeklējot atbilstošāko benzantrona luminoforu, tika noskaidrots, ka būtiska loma ir C-3 pozīcijā esošajam aizvietotājam, kas savā ziņā nosaka luminofora efektivitāti, atklājot strukturālo un vides ietekmi uz fotofizikāliem parametriem. Sintezētajiem luminoforiem ir plašs polaritātes diapazons (Kapusta et al., 2003; Siddlingeshwar et al., 2011; Shivraj et al., 2018), apstiprinot luminoforu izmantošanu dažādu bioloģisku struktūru vizualizēšanai (Kalnina et al., 2007; Trusova et al., 2012; Ryzhova et al., 2016; Kirjusina et al., 2018; Kirilova et al., 2019).

Benzantrona luminoforu izmantošanas laikā ir jārēķinās ar fotoizbalēšanas procesu, kā rezultātā pētāmā parauga kvalitāte sarūk ar katru reizi, kad uz to iedarbojas ar lāzeri (Han et al., 2021). Pētījumos (**I, II, III, IV**) apstiprinājās, ka detalizētu rezultātu iegūšanai, ir ieteicams lietot lāzera viļņa garumus dilstošā secībā. Fotoizbalēšanas process apstiprināja, ka ar benzantrona luminoforiem krāsotie un KLSM sagatavotie paraugi ir piemēroti ātrai un efektīvai parazītu sugu izpētei.

Promocijas darbā izvirzītā hipotēze, ka dažādu parazītu sugu efektīvai un ātrai izpētei ir piemērots noteikts krāsošanas protokols, izmantojot specifisku benzantrona luminoforu un konfokālo lāzerskenējošo mikroskopiju, tika apstiprināta.

**Trematodes (I, III)**

KLSM tiek plaši pielietota dažādu sugu morfoloģiskās un fizioloģiskās struktūras izpētē, it īpaši fiksētiem trematodes paraugiem. (Jurberg et al., 2008; Borges et al., 2017). Pētījuma eksperimentālie rezultāti (**I**) parādīja, ka, izmantojot 488 nm (ar 500-655 nm filtru) lāzera ierosmi, bija iespējams sasniegt 23x mazāku autofluorescences signālu, salīdzinājumā ar 405 nm (ar 425-580nm filtru) viļņa garuma ierosmi. Izvērtējot autofluorescenci, tika izvēlēti dažādi ROI un izvēlētie ROI tika salīdzināti ar fona ROI. Balstoties uz iegūtajiem datiem, 488 nm lāzeris ar FITC filtru (500-550 nm) un 638 nm lāzeris ar Cy5 filtru (662-737nm) bija vispiemērotākie lāzeri nevēlamas autofluorescences nomākšanai. Pētījumā ar saldūdens trematodēm (**III**) netika izmantots lāzeris ar 405 nm viļņa garumu, jo tas ierosina paraugu autofluorescenci. Pētījuma gaitā sintezētie benzantrona luminofori uzrādīja fluorescenci sarkanā spektra reģionā, tomēr ar benzantronu krāsotajiem

paraugiem bija fluorescences nobīde īsāka viļņa reģionā (**I, III**). Iespējams. hidrofobiskāku apstākļu dēļ (lielāks lipīdu skaits, dehidratēšana ar etanolu). Blakus luminiscenci var radīt ķīmiskais fiksators. Piemēram, izmantojot formalīnu saturošu ķīmiskā fiksatoru sajaukumu, var tikt novērota intensīvāka šūnu luminiscence spektra dzelteni - zaļā reģionā (Alfano et al., 1984).
Liekā ūdens klātbūtne šūnas struktūrās neļauj iegūt atbilstošu parauga caurspīdīgumu. Šigins (no angļu val. *Shigin*) (Shigin, 1996), krāsojot trematodes gaismas mikroskopijai, veica dehidratēšanas soli un arī pētījuma (**I, III**) rezultāti apstiprināja dehidratēšanas nozīmīgumu jaunā protokola izstrādē. Pētījuma rezultāti apstiprināja, ka parazītiem ar plānu apvalku struktūru, piem., Diplostomatidae dzimtai (**III**), nav vajadzības pielietot papildus dzidrināšanas soli, izmantojot 100% ksilēnu, jo absolūtais ksilēns var deformēt jutīgāku paraugu struktūru. Pētījuma laikā (**I**) tika novērots, ka trematodes biezums nosaka, cik ilgi paraugs ir jātur luminoforā un cik ilgi 100% ksilēnā. Novērojumi tika iekļauti krāsošanas protokola izstrādē, kur norādīts, ka no homoterma saimnieka izolētā trematode ir jāpatur piecas minūtes ilgāk luminoforā nekā no poikiloterma saimnieka izolēta trematode. *P. fasciolaemorpha* tiek turēts etanola:ksilēna šķīdumā 8-10 min, pēc tam 100% ksilēnā 30s – 3 min, šādas variācijas laikos ir pamatotas ar dažādos parazītu paraugu biezumu.
Trematodēm ir raksturīga ķermeņa sienas muskulatūras uzbūve, kas sastāv no trīs slāņiem: gredzeniskā, šķērssvītrotā un diagonālā (Ginetsinskaya, 1988; Galaktionov & Dobrovolskij, 2003). Izmantotā pētījuma materiālā (**I, III**) bija novērojami visi trīs raksturīgākie trematodes ķermeņa sienas muskulatūras slāņi. Muskuļu slāņu pētījumiem tiek izmantotas samērā sarežģītas krāsošanas metodes, kā piemēram, Krupenko (2014) parauga sagatavošana konfokālai lāzerskenējošai mikroskopijai prasīja divas dienas. Mūsdienu ritējumā divas dienas ir pārāk ilgs laiks, tāpēc tika meklētas iespējas izstrādāt krāsošanas protokolu, kas tērētu mazāk laika un cilvēka stundu resursu, bet sniegtu detalizētus rezultātus. Ar pētījumā izstrādātiem krāsošanas protokoliem rezultātus var iegūt jau pirmo divu stundu laikā (**I, II, III, IV**). Uzsākot pirmos mēģinājumus krāsot saldūdens trematodes, iegūtajos rezultātos netika novērota visa ķermeņa muskuļu struktūra, tomēr tika iezīmēta muskuļotā rīkle, mutes un vēdera piesūcekņi (**III**). Pētījumā ar *P. fasciolaemorpha* (**I**) tika novērota detalizētāka ķermeņa muskuļu struktūra, vizualizējot gredzeniskās, diagonālās un škērssvītrotās muskulatūras slāņus. Pētījumos (**I, III**) iegūtie rezultāti apstiprināja, ka sintezētie benzantrona luminofori un izstrādātie krāsošanas protokoli sniedza līdzīgus rezultātus kā krāsojot paraugus ar komerciāli pieejamo fluoresceīna izotiocianātu vai tetrametilrodamīna B izotiocianāts-konjugētu, kas iekrāso tieši aktīnu (Terentina et al., 2020). Pētījuma rezultāti (**I**) apstiprināja dzelksnīšu esamību uz ķermeņa virsmas, tāpat kā to apstiprināja citi pētnieki (Rankin, 1939; Belopolskaya, 1963; Davies, 1979; Saville et al., 1997; Pina et al., 2011; Krupenko & Dobrovolskij, 2018). Ar KLSM tika

novērots, ka ķermeņa priekšējā daļa ir blīvāk nosegta ar dzelkšņiem nekā ķermeņa apakšējā daļa (**I, III**). Lai gan Krupenko & Dobrovolskij (2018) secināja, ka dzelkšņu forma, zobiņu skaits utt. nav nosakāms tikai ar KLSM metodi, šī pētījuma rezultāti (**I**) apstiprināja pretējo. AZPP luminofors un KLSM metode sniedza informāciju par dzelkšņu izmēriem, zobiņu skaitu un to formu.

Pētījuma (**I**) rezultāti apstiprināja visu trīs raksturīgāko muskuļu slāņu esamību mutes piesūceknī: radiālie, ekvatoriālie (= gredzeniskie) un meridionālie (= šķērssvītrotie). Tika secināts, ka gredzeniskās muskuļu šķiedras ir blīvāk izvietotas tieši kopulācijas orgānā un kopulācijas orgāna maisiņā, salīdzinot ar *Fasciola hepatica* (Mair et al., 1998). Kopumā iegūtie pētījuma rezultāti (**I, III**) apstiprināja vispārējo trematodes ķermeņa morfoloģiju pēc Skrjabin (1949).

Zem tegumenta esošajās parenhīmas šūnās tika novērotas glikogēna rezerves, visvairāk tieši dzeltenuma dziedzeru zonā. Izkaisītas glikogēna rezerves tika novērotas piestiprināšanās orgānos. Visspilgtākā fluorescence tika novērota parazīta olās, kuras lielākoties sastāv no glikogēna un lipīdiem (**I**). Glikogēna rezerves un lipīdi kalpo par enerģijas avotu, šūnu aktivitātes regulatoru un to izmanto bioloģisko membrānu veidošanai (Swiderski et al., 2019).

Pētījumā izstrādātie krāsošanas protokoli (**I, III**) netika paredzēti kādas konkrētas orgānu sistēmas krāsošanai. Rezultātā, krāsojot trematodes ar sintezētajiem AZPP, AM323, AM1, AM2, AM4, AM16 un P8 benzantronu luminoforiem, netika novērota neviena no nervu sistēmas daļām.

Pētījumos tika izmantoti benzantrona luminofori ar trīs dažādām funkcionālām grupām. Luminoforu izvēle tika balstīta uz Kapusta et al. (2003); Siddlingeshwar et al. (2011); Shivraj et al. (2018) un Tarabara et al. (2021) pētījumiem, kur secināja, ka funkcionālā grupa nosaka luminofora efektivitāti, tādējādi pieļaujot varbūtību, ka pētījuma rezultātus varētu ietekmēt benzantrona funkcionālās grupas. Kopumā iegūtie rezultāti (**I, II, III, IV**) apstiprināja, ka luminofora funkcionālai grupai ir nozīme kvalitatīvu datu ieguvē. Efektīvākā parazītu izpēte tika veikta ar benzantroniem, kuriem ir aminoamido grupa (AZPP (**I**) un AZM (**II**)).

**Nematodes (II, IV)**

*Trichinella* ģints pētījumos KLSM lielākoties izmantota, pētot ekskretoro un sekretoro antigēnu (Li et al., 1999), imūnās atbildes (Bai et al., 2012), vai *T. spiralis* mijiedarbību muskuļu šūnās *in vitro* (Bai et al., 2011) un izmantota dažādos pētījumos, kur tiek izvērtēts patoloģiskais un/vai terapeitiskais efekts (Li et al., 1999; Masetti et al., 2004; Hao et al., 2014). *T. spiralis* un *T. britovi* parazītu sugas ir cilvēkam un dzīvniekam bīstamās trihinelozes ierosinātājas (Rozycki et al., 2022; Tso et al., 2022).

Parasti autofluorescences ierosmes reģions atrodas apmēram 400 nm un pētījumā tika izvēlēta 488 nm absorbcija (**II**), slāpējot autofluorescences signālu attiecībā pret marķiera fluorescences signālu (Schnell et al., 1999;

Neumann & Gabel, 2002). Absorbcija pie 488 nm deva labāko fluorescences/autofluorescences signālu attiecību (**II, IV**).
Pētāmo paraugu datu kvalitāti ietekmē pētāmā paraugu uzglabāšanas apstākļi. Trihinellu kāpuri tāpat kā trematodes paraugi (**I, III**) tika fiksēti vairākos ķīmiskos fiksatoros un tika apstiprināts, ka ķīmiskais fiksators ietekmē gala rezultātu. Pētījums (**II**) apstiprināja, ka vispiemērotākais ķīmiskais fiksators trihinellu kāpuru efektīvai izpētei ir Bouina fiksators.
Pētījuma ietvaros izstrādātais krāsošanas protokols ir piemērots nesen ievāktiem, no dzīvnieku muskulatūras izdalītiem un fiksētiem trihinellu kāpuru paraugiem (**II**). Izstrādātie protokoli ir ātri un vienkārši (**II, IV**) salīdzinot ar ražotāja izstrādātiem standarta krāsošanas protokoliem, kur paraugu sagatavošana sastāv no vairākiem soļiem, kur viens solis var ilgt pat vairākas stundas (Stewart et al., 2003a,b,c; Davila et al., 2010).
Viena no pazīmēm ar ko var noteikt trihinellu kāpuru dzimumu ir gala zarnas garums. Tēviņiem vidējais gala zarnas garums ir 40 µm līdz 50 µm, bet mātītēm tas ir gandrīz uz pusi mazāks 17 µm līdz 35 µm. Pētījuma laikā tika izstrādāts krāsošanas protokols (**II**), kas ir piemērots kāpura dzimuma noteikšanai, izmērot gala zarnas garumu. Pētījuma rezultātos tika iegūts, ka gala zarnas garums *T. britovi* tēviņiem ir 41.08 ± 4.26 µm SD un *T. spiralis* - 46.08 ± 2.95 µm SD; mātītēm 21.19 ± 2.45 µm SD un 20.55 ± 1.48 µm SD. Iegūtie pētījuma dati sakrīt ar citu pētījumu datiem un apstiprina, ka tēviņu gala zarnas garums ir divreiz lielāks nekā mātītēm (Kozek 1975; Liu et al., 1991, Pozio & La Rosa, 2010).
Pētījumā iegūtie rezultāti apstiprināja tipisko nematodes ķermeni (**II, IV**): šaurāka ķermeņa priekšējā daļa un platāka ķermeņa aizmugurējā daļa. Kāpuru kutikula sastāv no trīs vai vairāk ārējiem slāņiem, kas ir veidoti no kolagēna un citām sastāvdaļām (Lichtenfels et al., 1983). Iegūtie rezultāti uzrādīja augstu fluorescences signālu kāpuru kutikulā (**II, IV**). Augstā fluorescences signāla cēlonis kutikulā var būt lipīdu uzkrāšanās trihinellu kāpuru epikutikulā (Gounaris et al., 1996). Kopumā trihinellas ķermeni klāj rievota kutikula (Hetherington, 1924) un šī pētījuma laikā tika novērots, ka pastāv atšķirības starp *T. spiralis* un *T. britovi* kāpuru kutikulu. *T. spiralis* kāpuriem tika novērots rievojums, tā saucamā "pseidosegmentācija", savukārt *T. britovi* kutikulā netika novērotas šķērsas līnijas vai rievojums (**II**). Muskuļu kāpuram ir pietiekami attīstīti orgāni (McVay et al., 1998). Pētījumos apstiprinājās, ka muskuļu kāpuram ir attīstīta gan gremošanas, gan reproduktīvā orgānu sistēma (**II, IV**). *Stichosome* un stihocīti tika novēroti gan sasaldētos paraugos, krāsoti ar P8 (**IV**), gan no muskuļiem izdalītos kāpuros, krāsoti ar AZM (**II**). Krāsojot paraugus ar AZM (**II**), tika novērots barības vads, kas sastāv no vienslāņa epitēlija ar bazālo membrānu bazālajā pusē. Zinātniskie pētījumi ir apstiprinājuši, ka trihinellu barības vadā ir novērojami četri epitēliju tipi un dažas no epitēlija šūnām ir mioepitēlijs, kas nodrošina barības vada peristaltiku (Takahashi, 2021). Pētījumos (**II, IV**) netika novērotas dažādas epitēliju šūnas,

bet pastāv iespēja, ka, optimizējot krāsošanas protokolu, tās varētu novērot. Ar AZM krāsotajos paraugus tika novērota gan viduszarna, gan dzimumorgānu aizmetnis (**II**), bet ar P8 – tikai dzimumorgānu aizmetnis (**IV**). Ar AZM tika iekrāsotas visa gremošanas orgānu sistēma (**II**). Vēl viena pazīme, kas var atšķirt tēviņu no mātītes kāpura ir glikogēna krājumu daudzums dzimumorgānu aizmetnī (Takahashi et al., 1987). Mātītēm šie krājumi ir lielāki, līdz ar to, krāsojot paraugus ar benzantrona luminoforiem, vietā, kur būs lielāks glikogēna daudzums, būs novērojama intensīvāka fluorescence. Rezultātu izvērtēšanā tika secināts, ka glikogēna krājumus mātītes kāpuros labāk iekrāso P8 (**IV**).

Pētījumā ar AZM luminoforu tika novērots nervu gredzens, bet netika novērotas detalizētākas struktūras. Ar P8 luminoforu (**IV**) netika vizualizētas nervu sistēmas daļas.

Kopumā tika sintezēti deviņi benzantrona luminofori *P. fasciolaemorpha* (ad.) (AM323, AZPP), *D. spathaceum* (mtc.), *D. subclaviatus* (ad.) un *P. confusus* (ad.) (AM1, AM2, AM4, AM16, P8), *T. spiralis* un *T. britovi* (AZM, P13) efektīvai un ātrai izpētei. Apkopojot iegūtos rezultātus, jāsecina, ka benzantroni ar aminoamido fukcionālo grupu sniedza viskvalitatīvākos rezultātus gan Trematoda, gan Nematoda parazītu sugu izpētē. Tika izstrādāti noteikti krāsošanas protokoli parazītu sugu efektīvai un ātrai izpētei, izmantojot KLSM, iegūstot detalizētus rezultātus divu stundu laikā. Protokolu izstrādes laikā tika novērots, ka krāsošanas soļa ilgumu trematodes paraugiem nosaka tas, no kāda saimnieka (poikiloterma vai homoterma) trematode tikusi iegūta. Krāsošanas protokoli var tikt izmantoti paraugiem, kas fiksēti septiņos dažādos ķīmiskos fiksatoros, kā arī trīs dažādu tipu trihinellu kāpuru parazītiem: vienas dienas laikā no dzīvnieku muskulatūras izdalītiem un fiksētiem kāpuriem; kāpuriem, kas uzglabāti 96% etanolā un kāpuriem sasaldētā dzīvnieku muskulatūrā. Izmantojot specifisku benzantrona luminoforus un noteiktus krāsošanas protokolus, ir iespējams noteikt atšķirības *T. spiralis* un *T. britovi* kutikulas uzbūvē un precīzi noteikt abu trihinellu kāpuru dzimumu.

**SECINĀJUMI**

1. Kopumā tika aprobēti deviņi sintezētie benzantrona luminofori Trematoda klases un Nematoda tipa parazītu sugu efektīvai un ātrai izpētei (**I, II, III, IV**).
2. *P. fasciolaemorpha* (ad.) (**I**), *D. spathaceum* (mtc.), *D. subclaviatus* (ad.), *P. confusus* (ad.) (**III**) parazītu, kas fiksēti 70% un 96% etanolā; AFA, Karnoja un Bouina šķīdumā; 10% neitrāli buferētā formalīnā, sugu efektīvai un ātrai izpētei tika izstrādāti krāsošanas protokoli, izmantojot AM323, AZPP, AM1, AM2, AM4, AM16 un P8 benzantrona luminoforus. *P. fasciolaemorpha* parazītu paraugu anatomiskās un muskulatūras izkārtojuma detalizētai izpētei ar AZPP luminoforu vispiemērotākie ir 70% etanolā vai AFA šķīdumā fiksēti paraugi: 70% etanols un AZPP luminofors ārējo struktūru un muskuļu slāņu izpētei; AFA šķīdums un AZPP luminofors iekšējo struktūru izpētei. Bouina fiksators nav piemērots *P. fasciolaemorpha* paraugu fiksēšanai, ja tie tiek analizēti ar KLSM (**I**). AM1, AM2, AM4, AM16 un P8 luminofori dod iespēju detalizēti vizualizēt gremošanas un reproduktīvo sistēmu *D. spathaceum* (mtc.), fiksētiem 96% etanolā; *D. subclaviatus* (ad.), fiksētiem Karnoja šķīdumā; *P. confusus* (ad.), fiksētiem AFA šķīdumā (**III**). Tika secināts, ka no poikiloterma saimnieka izolētas trematodes krāsošanai ir vajadzīgs mazāk laika nekā no homoterma saimnieka izolētas trematodes krāsošanai.
3. *T. spiralis* un *T. britovi* parazītu, kas fiksēti 70% un 96,6% etanolā; AFA, Karnoja un Bouina šķīdumā sugu efektīvai un ātrai izpētei tika izstrādāti krāsošanas protokoli, izmantojot AZM un P13 benzantrona luminoforus. Tika secināts, ka sintezētais benzantrona luminofors AZM ir piemērots no dzīvnieku muskulatūras izolētiem trihinella kāpuriem, fiksētiem dažādos ķīmiskos fiksatoros, izpētei. Izstrādātais krāsošanas protokols ir piemērots *T. spiralis* un *T. britovi* kāpuru dzimumu noteikšanai, mērot kāpuru gala zarnas garumu (**I**). Sasaldētā dzīvnieku muskulatūrā vai 96,6% etanolā uzglabātu *T. britovi* kāpuru efektīvai un ātrai izpētei tika izstrādāts krāsošanas protokols ar P13 (**IV**).
4. Trematoda klases un Nematoda tipa parazītu sugu efektīvai un ātrai izpētei izstrādāti noteikti krāsošanas protokoli, izmantojot specifiskus benzantrona luminoforus. Atrasti piemērotākie ķīmiskā fiksatora un benzantronu luminofora kompleksi paraugu izpētei, pielietojot KLSM: *P. fasciolaemorpha* (ad.) 70% etanols vai AFA šķīdums un AZPP (**I**); *T. spiralis* un *T. britovi* Bouina šķīdums un AZM (**II**); *D. spathaceum* (mtc.) 96% etanols, *D. subclaviatus* (ad.) Karnoja šķīdums, *P. confusus* (ad.) AFA šķīdums un AM1, AM2, AM4, AM16 un P8 (**III**); *T. britovi* 96,6% etanolā līdz trīs gadiem vai saldēti kāpuri dzīvnieku muskulatūrā līdz diviem gadiem un P13 (**IV**).

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DAUGAVPILS UNIVERSITY
INSTITUTE OF LIFE SCIENCES AND TECHNOLOGY

ILZE RUBENIŅA

# BENZANTHRONE LUMINOPHORES FOR EFFECTIVE AND RAPID STUDY OF TREMATODA AND NEMATODA PARASITES

SUMMARY

Of the Doctoral Thesis in Biology
for the Scientific Degree (Ph.D)
(Branch: Zoology)

DAUGAVPILS 2022

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**Type of work:** Doctoral Thesis (a set of publications) in Biology, branch Zoology.

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**The defence of the Doctoral Thesis** will take place in Daugavpils University (Parades street 1A) an open meeting of the Promotion Council of Biological Science on 2022. …, at … AM/PM, online at Zoom platform/ Room 130.

The Doctoral Thesis and its summary are available at the Library of Daugavpils University, Parādes street 1 in Daugavpils, Latvia and from www.du.lv.

**The comments are welcome**. Send them to the secretary of the Promotion Council, Parādes street 1A, Daugavpils, Latvia, LV-5401, Tel. +371 260 02 593, e-mail: jana.paidere@du.lv.

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## LIST OF ORIGINAL PAPERS

The thesis is based on the following papers, which are referred to in the text by Roman numerals. Original papers are reproduced with permissions from the publishers.

I. Rubenina I., Gavarane I., Kirilova E., Mezaraupe L., Kirjusina M. (2021) Comparison of the Benzanthrone Luminophores: They Are Not Equal for Rapid Examination of *Parafasciolopsis fasciolaemorpha* (Trematoda: Digenea). Biomolecules 11(598):1-15. https://doi.org/10.3390/biom11040598

II. Gavārane I., Kirilova E., Rubeniņa I., Mežaraupe L., Osipovs S., Deksne G., Pučkins A., Kokina I., Bulanovs A., Kirjušina M. (2019) A Simple and Rapid Staining Technique for Sex Determination of *Trichinella* Larvae Parasites by Confocal Laser Scanning Microscopy. Microscopy and Microanalysis, 1-7. doi:10.1017/S1431927619015046

III. Kirilova E., Kecko S., Mežaraupe L., Gavarāne I., Pučkins A., Mickeviča I., Rubeniņa I., Osipovs S., Bulanovs A., Pupiņš M., Kirjušina M. (2018) Novel luminescent dyes for confocal laser scanning microscopy used in Trematoda parasite diagnostics. Acta Biochimica Polonica 65(3):449-454. https://onlinelibrary.wiley.com/doi/abs/10.1002/bio.3616

IV. Gavarane I., Mezaraupe L., Rubenina I., Kirjusina M., Kirilova J. (2018). Staining of economically important parasitic nematodes by developed derivatives of benzanthrone luminophore. 18th International Multidisciplinary Scientific GeoConference SGEM 2018, section Advances in Biotechnology, 581–587. https://doi.org/10.5593/sgem 2018/6.2

The author's contribution (%) to the papers:

| | **I.** | **II.** | **III.** | **IV.** |
|---|---|---|---|---|
| Original idea | 50 | 20 | 30 | 30 |
| Study design | 50 | 30 | 30 | 40 |
| Data collection | 60 | 50 | 30 | 50 |
| Data analysis | 80 | 50 | 40 | 60 |
| Manuscript preparation | 90 | 50 | 60 | 50 |

# INTRODUCTION

## Topicality of the study

With the development of the microscopy, it significantly changed the course of biology studies. The microscope is used in the study of various life processes, thus characterizing the microscopic structure of organisms. The confocal laser scanning microscopy method is one of the methods to study the internal and external structure of parasites, obtaining detailed quantitative and qualitative data (Jurberg et al., 2008; Kirjusina et al., 2018; Terenina et al., 2018; Mochalova et al., 2019; Terenina et al., 2020).

Helminth species are involved in competitive and predatory interactions with both host and non-host taxons, thus showing the importance of parasites in ecosystems and food webs (Tompkins et al., 2011). Trematoda class and representatives of the genus *Trichinella* (Nematoda phylum) are initiators of various zoonoses (Sah et al., 2020). Trematoda class includes representatives of schistosomes and food-borne trematodes who have infected more than 294 million people worldwide (*World Health Organisation*, 2015, 2019; Bennett & Robinson, 2021). In turn, the zoonosis caused by the *Trichinella* nematode parasite – trichinelosis is ranked in the top 10 of the world's most dangerous diseases for humans (Pozio & Zarlenga, 2019). According to the World Health Organization, 10,000 people each year test positive for *Trichinella* infection (*World Health Organization*, 2022).

Using fluorescent microscopy, luminophores are given great importance (Ndao, 2009). In the past, fluorescent dyes like acridine orange and rhodamine C (Geller & Timonov, 1969a,b; Stankiewicz et al., 1996; Janssen 1998) were widely used for the staining of the structure of parasites, but nowadays other synthesized dyes for morphological study of biological organisms gradually began to appear (Dapson, 2007; Fakhar & Ghobaditara, 2016). Benzanthrone luminophores have gained popularity in recent years and their use makes it possible to identify specific lipids and proteins (Zhytniakivska et al., 2014a; Ryzhova et al., 2016). Benzanthrone luminophores are known as fluorescent probes, which, depending on the structure of color, emit in a certain spectral region: yellow - green or red - purple (Khrolova et al., 1984). Some benzanthrone luminophores are used to monitor structural changes in membranes and to study the pairing of nucleic acids (Dobretsov, 1989; Yang et al., 1999). Studies have confirmed that luminophores can be used to stain biological objects. Although staining with benzanthrone luminophores greatly reduces the time of preparation of samples, each group of organisms should be matched with a specific benzanthrone luminophore (Kirjusina et al., 2018; Kirilova et al., 2019).

Trematoda sample preparation protocols for confocal laser scanning microscopy, described in the studies, take more than one (Krupenko, 2014) or even more than two days (Krupenko, 2014; Krupenko & Dobrovolskij; 2018;

Krupenko, 2019) and is focused on the study of certain systems, such as the muscular system (Krupenko, 2019), the nervous system (Kremnev et al., 2020, 2021). Various sample washing and fixing solutions are used during the sample preparation phase, such as paraformaldehyde, phosphate buffer solution or PBS with 0.1% sodium azide, etc. (Krupenko & Gonchar, 2017a,b; Krupenko et al., 2016). As a result, the protocols require plenty of resources and hours of human work. *Trichinella* staining protocols, on the other hand, are often not intended to determine the structure of the larvae itself, but to monitor specific processes such as immune system response (Bai et al., 2012), testing of anti-*Trichinella* vaccines (Hu et al., 2021), localization of certain proteins (Hernandez-Bello et al., 2008; Morales-Montor, 2022), study of interactions between nematodes and muscle cells *in vitro* (Bai et al., 2011). However, there is a lack of luminophores that are capable of staining the general structure of parasites without spending a lot of resources and time preparing a sample for confocal laser scanning microscopy.

Microscopic research of the parasites includes sex determination. One of the characteristics of populations is gender structure. Kozek (1975) and Li et al. (1999) have attempted to determine the sexes of *Trichinella* larvae by listing the morphological characteristics that distinguish a female from a male. However, attempts have not been successful, because, when examining samples with light microscopy, it is relatively difficult to distinguish between characteristics that require great accuracy (Weller, 1943; Kozek, 1975). One of the attributes by which it is possible to determine the sex of the larvae is measuring the length of the rectum, however, measurements obtained by light microscopy may be inaccurate (Villella, 1966).

**Novelty of the research**

Protocols for the study of helminths described in studies of trematode parasite species using confocal laser scanning microscopy mostly are standard protocols developed by manufacturers with minor modifications, which require more than one or even more than two days for the preparation of samples (Krupenko, 2014; Krupenko & Dobrovolskij; 2018; Krupenko, 2019), in addition, staining protocols are focused on the study of specific organ systems (Krupenko, 2018; Kremnev et al., 2020, 2021). In turn, *Trichinella* staining protocols are aimed at observing specific processes or localizing certain proteins (Hernandez-Bello et al., 2008; Bai et al., 2011, 2012; Hu et al., 2021; Morales-Montor, 2022). However, there is a lack of luminophores and staining protocols that would focus on the parasite's structure itself without spending excessive laboratory resources and time on preparing the sample for confocal laser scanning microscopy.

In the course of this study, seven benzanthrone luminophores (AM323, AZPP, AM1, AM2, AM4, AM16, P8,) were synthesized and certain staining protocols were developed for the study of different species of Trematoda class parasites

using confocal laser scanning microscopy. The developed staining protocols are suitable for effective and rapid *P. fasciolaemorpha* (ad.), *D. spathaceum* (ad.), *D. subclaviatus* (mtc.) and *P. con*fusus (ad.) research. Two benzanthrone luminophores (AZP and P13) were synthesized and certain staining protocols were developed for the study of Nematoda phylum parasite species. The developed staining protocols are suitable for studying three different types of *Trichinella* samples: samples stored in 96.6% ethanol, frozen larvae in animal musculature or recently collected animal musculature, from which *Trichinella* larvae have been isolated within a day and fixed. As a result, morphological differences were observed between samples of the *T. spiralis* and *T. britovi* species. During the study, AZM luminophore was synthesized and a certain staining protocol was developed for determining the sex of *T. spiralis* and *T. britovi* larvae based on the length of the larva's final intestine.
By selecting a specific benzanthrone luminophore for different species of parasites, the effectiveness of the developed protocols was increased.
The developed staining protocols save the time and laboratory resources of sample preparation, since it is not necessary to spend several hours or days to prepare samples for confocal laser scanning microscopy.

**Hypothesis**
For the effective and rapid research of different species of parasites a certain staining protocol is determined, using a certain chemical fixative and a specific benzanthrone luminophore for confocal laser scanning microscopy.

**The aim of the study**
To develop a staining protocol using benzanthrone luminophores for effective and rapid study of different species of Trematoda class and Nematoda phylum using confocal laser scanning microscopy.

**Tasks of the study**
1. To approbate the synthesized benzanthrone luminophores for effective and rapid study of various species of parasites of Trematoda class and Nematoda phylum (**I, II, III, IV**).
2. To develop staining protocol using a specific benzanthrone luminophore, for effective and rapid study of Trematoda parasite species fixed in various chemical fixatives (**I, III**).
3. To develop a staining protocol using a specific benzanthrone luminophore, for effective and rapid study of three different types of Nematoda parasite species fixed in different chemical fixatives and for sex determination (**II, IV**).
4. To analyse the suitability of the synthesized benzanthrone luminophore and the effectiveness of the staining protocol for effective and rapid study of Trematoda class and Nematoda phylum parasite species using confocal laser scanning microscopy (**I, II, III, IV**).

**Statements to be defended**

1. The synthesized benzanthrone luminophores are suitable for effective and rapid studying of different species of parasites of Trematoda class and Nematoda phylum using confocal laser scanning microscopy.
2. Chemical fixatives affect the detailed study of the sample being studied and a certain fixative is suitable for a specific luminophore.
3. The duration of Trematoda parasite staining step is influenced by the origin of the sample – isolated from poikilotherm or homotherm host.
4. Development of an effective and rapid staining protocol provides detailed results within two hours.
5. A specific benzanthrone luminophore and a certain staining protocol are suitable for determination of the sex of *T. spiralis* and *T. britovi* larvae.

**Approbation of the research results**

*Scientific monography:*

The contribution of all authors to the creation of the monograph is the equal, irrespective of the order of mention of authors.

Gavarane I., Kirilova E., Rubenina I., Osipovs S., Mezaraupe L., Puckins A., Kirjusina M. 2020. Simple and rapid luminiscent staining protocols in Helmintology. [Monograph]. Published by Daugavpils University "Saule", Daugavpils, 131 pages.

*Patent:*

Gavarāne I., Rubeniņa I., Kirilova J., Kirjušina M. Luminescent method for sex determination of the *Trichinell*a spp. parasite larva. Publication of the patent application. Official edition of the Patent Office of the Republic of Latvia. Inventions, trademarks and designs 2020-03. Patent Office of the Republic of Latvia, 2020. ISSN 2255-9655 patent filed on 20.12.2019. (Application No. P-19-76). Patent publication date 20.03.2021., Patent register No. 15489.

The results of the research have been presented at *several international conferences:*

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2. Bordjuga V., Mežaraupe L., Rubeniņa I., Kirjušina M. 2021. Development of staining protocol using benzanthrone luminophores for trematodes muscle research. Abstracts of the 63 Scientific conference of Daugavpils University, Daugavpils, Latvia, 15-16 April, Abstract Book, p. 30 (*verbal presentation*)
3. Širokova J., Mežaraupe L., Rubeniņa I., Kirjušina M. 2021. Comparison of different fluorescent dyes for trematoda *Prototocus confusus* imaging. Abstracts of the 63 Scientific conference of Daugavpils University, Daugavpils, Latvia, 15-16 April, Abstract Book, p. 28 (*verbal presentation*)

4. Rubenina I., Gavarane I., Mezaraupe L., Gravele E., Pupins M., Kirjusina M. 2021. Description of *Prosotocus confusus* in *Phelophylax esculentus* complex and *Rana temporaria* from Latvia. Abstract book, 79. scientific conference Zoology and Animal Ecology, Univerity of Latvia, Riga, Latvia, January 28, 2021, Abstract Book p. 26 (*verbal presentation*)
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6. Gavarāne I., Rubeniņa I., Mežaraupe L., Kirilova E., Kirjušina M. 2019. Luminophores for *Trichinella britovi* larvae examination and sex determination using confocal laser scanning microscopy. October 09-11, 2019. Copenhagen, Denmark, the 8th Conference of the Scandinavian- Baltic Society for Parasitology (SBSP) and the Annual Meeting of the European Veterinary Parasitology College (EVPC), Abstract Book, p. 64 (*poster presentation*)
7. Rubeniņa I., Mežaraupe L., Gavarāne I., Kirilova E., Kirjušina M. 2019. Perspectives in studies of adult *Parafasciolopsis fasciolaemorpha* by confocal laser scanning microscopy. October 09-11, 2019. Copenhagen, Denmark, the 8th Conference of the Scandinavian-Baltic Society for Parasitology (SBSP) and the Annual Meeting of the European Veterinary Parasitology College (EVPC), Abstract Book, p. 30 (*verbal presentation*)
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9. Gavarāne I., Rubeniņa I., Kirilova E., Kirjušina M. 2019. Study of fish parasites *Dactylogyrus* by confocal laser scanning microscopy. 3-6 October 2019. Jahorina, Bosnia and Herzegovina, X International Agriculture Symposium "AGROSYM 2019", Book of Abstracts, 716 pages (*poster presentation*)
10. Kirjušina M., Rubeniņa I., Kirilova E., Gavarāne I. 2019. Benzanthrone luminophores for examination of fish *Diplostomum* metacercariae. 3-6 October 2019. Jahorina, Bosnia and Herzegovina, X International Agriculture Symposium "AGROSYM 2019", Book of Abstracts, 719 pages (*poster presentation*)
11. Gavarāne I., Rubeniņa I., Mežaraupe L., Bulanovs A., Kirjušina M., Kirilova J. 2019. Novel benzanthrone luminophore AZM for *Trichinella britovi* larvae sex determination. 17-19 June 2019. Riga, Latvia, FEBS3+ Conference (*poster presentation*)

12. Kirjušina M., Gavarāne I., Mežaraupe L., Rubeniņa I., Kecko S., Kirilova E. 2019. Application of novel synthesized luminophores for staining of adult Trematoda in confocal laser scanning microscopy. May 12-18, 2019, Oludeniz, Turkey, INTERIM 2019 6th International congress on microscopy & spectroscopy (*poster presentation*)
13. Mežaraupe L., Kecko S., Kirilova E., Gavarāne I., Kirjušina M., Rubeniņa I. 2019. Staining Of Trematode *Parafasciolopsis Fasciolaemorpha* (Fasciolidae) With Luminiscent Benzanthrone AM323. 02-04 May 2019. Kaunas, Lithuania, 3rd International Conference „Smart Bio", Book of Abstracts, 213 pages (*poster presentation*)
14. Gavarāne I., Rubeniņa I., Mežaraupe L., Kokina I., Kirilova E., Kirjušina M. 2019. Molecular identification of *Trichinella* spp. and sex ratio determination of parasite larvae obtained from red fox. 11-12 April 2019, Daugavpils, Latvia, 61st International Scientific Conference of Daugavpils University, Book of Abstracts, p. 13 (*verbal presentation*)
15. Gavarāne I., Kirilova E., Kirjušina M., Kecko S., Plaksenkova I., Mežaraupe L., Rubeniņa I. 2018. *Trichinella* spp. identification by molecular biology methods and larvae examination by fluorescence microscopy techniques with developed benzanthrone luminophores. October 24 – 27, 2018, Riga, Latvia, 7th Baltics genetics congress, Environmental and Experimental Biology 16, Book of Abstracts, p. 206. https://doi.org/10.22364/eeb.16.18 (*poster presentation*)
16. Osipovs S., Kirilova E., Kirjusina M., Gavarane I., Rubenina I., Mezaraupe L., Puckins A. 2018. Application of novel synthesized luminophores for microscopic visualization of biological objects. 03-06 September 2018. Caparica, Portugal, 3rd International Caparica Conference on Chromogenic and Emissive Materials (*poster presentation*)
17. Gavarane I., Mezaraupe L., Rubenina I., Kirjusina M., Kirilova J. 2018. Staining of economically important parasitic nematodes by developed derivatives of benzanthrone luminophore. June 30-July 9, Albena, Bulgaria, 18th International Multidisciplinary Scientific GeoConference SGEM, section Advances in Biotechnology, Book of Abstracts, p. 581–587 (*poster presentation*)
18. Gavarāne I., Mežaraupe L., Kecko S., Deksne G., Rubeniņa I., Kirjušina M., Kirilova J. 2018. Using new fluorophore AZM for staining *Trichinella spiralis* for confocal laser scanning microscopy. 03-05 May 2018. Kaunas, Lithuania, 2nd International Conference „Smart Bio", Book of Abstracts, 285 pages, ISBN 978-609-8104-48-6 (*poster presentation*)
19. Kirjušina M., Pučkins A. Mickeviča I., Kirilova J., Osipovs S., Rubeniņa I., Jahundoviča I. 2017. Diagnostic of parasites using novel luminescent dyes and confocal laser scanning microscope. 11- 14 September 2017, Krakow, Poland, EUROBIOTECH 6th Central Europe Congress of Life Sciences Eurobiotech, Book of Abstracts, 128 pages (*poster presentation*)

*Popular Scientific papers:*

1. Gavārane I., Kirilova E., Rubeniņa I., Mežaraupe L., Osipovs S., Deksne G., Pučkins A., Kokina I., Bulanovs A., Kirjušina M. 2020. A Simple and Rapid Staining Technique for Sex Determination of *Trichinella* Larvae Parasites by Confocal Laser Scanning Microscopy. Highlights from Microscopy and Microanalysis. Microscopy Today, 28(2):58-59. doi:10.1017/S1551929519001378
2. Gavarāne I., Rubeniņa I., Kirilova J., Kirjušina M. 2018. Christmas Worm or Meat Parasite *Trichinella* [in Latvian]. Medības 12(77):62-64
3. Jahundoviča I., Mickeviča I., Kalniņa I., Rubeniņa I., Kirilova J., Kirjušina M. 2017. Topicalities in Diagnostics Fluorescence *in situ* Hybridization Method for Diagnosis of Parasites Dangerous to Human Health [in Latvian]. Latvijas ārsts 8:32-37

# MATERIALS AND METHODS

### Description of the synthesis of benzanthrone luminophores (I, II, III, IV)

Nine benzanthrone luminophores were synthesized in the Department of Applied Chemistry of the Institute of Life Sciences and Technologies of Daugavpils University (DU LSTI): AZPP, AM323, AZM, AM1, AM2, AM4, AM16, P8 and P13, which were used in the staining of biological objects during the course of the study and the development of specific staining protocols for effective and rapid study of Trematoda class and Nematoda phylum parasites of different species.

### Research material (I, II, III, IV)

During the research, helminth samples from the Department of Parasitology and Histology of the Department of Ecology of the DU LSTI were used. Helminths were obtained from various hosts: mice (**II**), red fox (**II, IV**), elk (**I**), fish (**III**), amphibians (**III**). The description of the research material is shown in Figure 1.

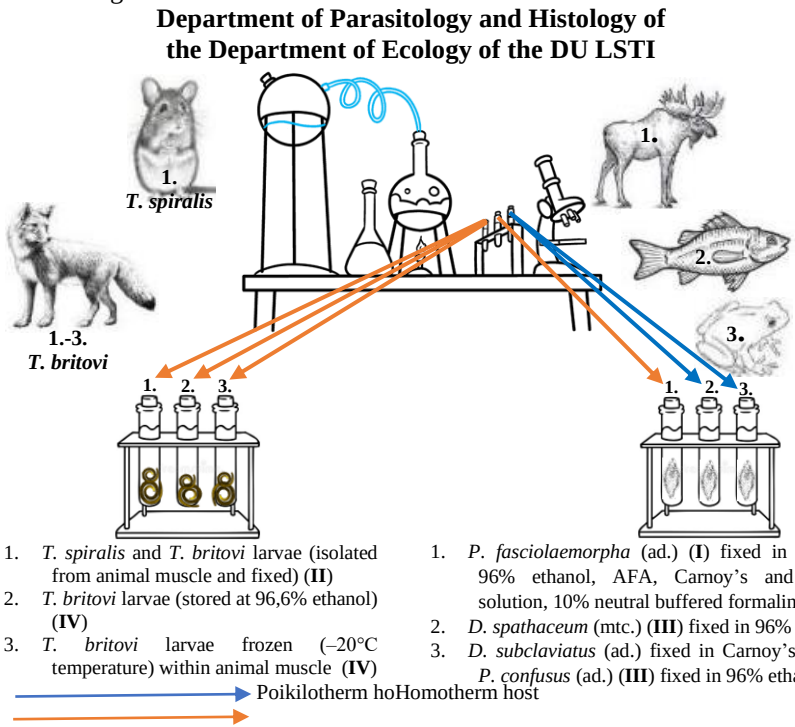


**Figure 1.** Research material used in the research

**Chemical fixation of the material under the research (I, II, III, IV)**

Immediately after isolation from the host organism, samples of trematode parasite species were chemically fixed in six different fixatives (Table 1).

Table 1

**Chemical fixation and storage of samples of Trematoda parasite species**

| Chemical fixative | 70% ethanol (**I**) | 96 % ethanol (**I, III**) | AFA solution (**I, III**) | Carnoy's solution (**I**) | Bouin's solution (**I**) | 10% neutral buffered formalin (**I**) |
|---|---|---|---|---|---|---|
| Time of fixation *P. fasciolaemorpha* | Until examination | Until examination | 2 h | 2 h | 2 h | Until examination |
| Time of fixation for freshwater trematodes | - | Until examination | 1 h | 1 h | - | - |
| Washing of *P. fasciolaemorpha* | Not applicable | Not applicable | 70% ethanol | 70% ethanol | 70% ethanol | Not applicable |
| Washing of freshwater trematodes | - | Not applicable | 96 % ethanol | 96 % ethanol | - | - |
| Chemical fixative where samples of *P. fasciolaemorpha* were stored | 70% ethanol | 96 % ethanol | 70% ethanol | 70% ethanol | 70% ethanol | 10% neutral buffered formalin |
| Chemical fixative where samples of freshwater trematodes were stored | - | 96 % ethanol | 96 % ethanol | 96 % ethanol | - | - |
| Storage | Refrigerated at +4°C temperature until required | | | | | |

- chemical fixative was not used for sample fixation

Isolated specimens of *T. spiralis* and *T. britovi* larvae immediately after isolation from the muscles of the animals were fixed for 1 hour in four different chemical fixatives (Table 2).

Table 2

**Chemical fixation and storage of samples of *T. spiralis* and *T. britovi* parasite species**

| Chemical fixative | 70% ethanol (**II**) | 96.6 % ethanol (**IV**) | AFA solution (**II**) | Carnoy's solution (**II**) | Bouin's solution (**II**) |
|---|---|---|---|---|---|
| Time of fixation | Until examination | Until examination | 1 h | 1 h | 1 h |
| Washing | Until examination | Until examination | 70% ethanol | 70% ethanol | 70% ethanol |
| Chemical fixative where samples were stored | 70% ethanol | 96.6% ethanol | 70% ethanol | 70% ethanol | 70% ethanol |
| Storage temperature | Refrigerated at +4°C until required | Refrigerated at +4°C until required | Refrigerated at +4°C until required | Refrigerated at +4°C until required | Refrigerated at +4°C until required |
| Time of storage | Day to 1 year | 1 – 5 years | Day to 1 year | Day to 1 year | Day to 1 year |

**Staining protocols with specific benzanthrone luminophore (I, II, III, IV)**

Benzanthrone luminophores AZPP and AM323 were synthesized for *P. fasciolaemorpha* samples fixed in 70% and 96% ethanol, AFA, Carnoy's and Bouin's solutions or 10% neutral buffered formalin (**I**). The parasite was isolated from the elk (*Alces alces*) (homothermic host). The overall staining process of the Trematoda samples is shown in Figure 2.

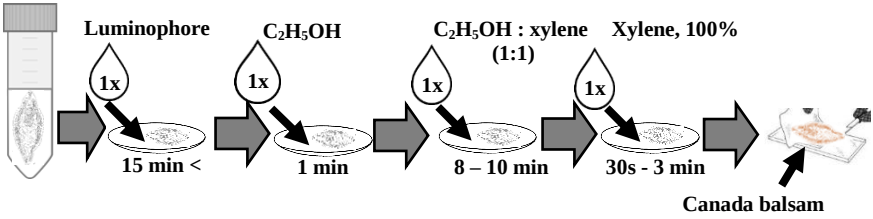


**Figure 2.** General staining process of Trematoda samples (Rubeniņa, 2022)

Benzanthrone luminophores AM1, AM2, AM4, AM16 and P8 were synthesized for freshwater trematodes: *D. spathaceum* (mtc.), fixed in 96% ethanol; *D. subclaviatus* (ad.) fixed in Carnoy's solution and *P.confusus* (ad.) fixed in AFA solution (**III**). The developed staining protocol was applied to samples from poikilothermic hosts: amphibians (*D. subclaviatus*, *P. confusus*) and freshwater fish (*D. spathaceum)*.

Benzanthrone luminophore AZM was synthesized for trichinella larvae isolated from animal muscle and fixed in 70% ethanol, Carnoy's, Bouin's or AFA solution. In turn, luminophore P13 was synthesized for the larvae of *T. britov*i, some of which were fixed in 96.6% ethanol from 1 to 5 years, and the second part of the larvae were frozen in animal musculature from 1 to 5 years. The overall staining process of the Nematoda samples is shown in Figure 3.

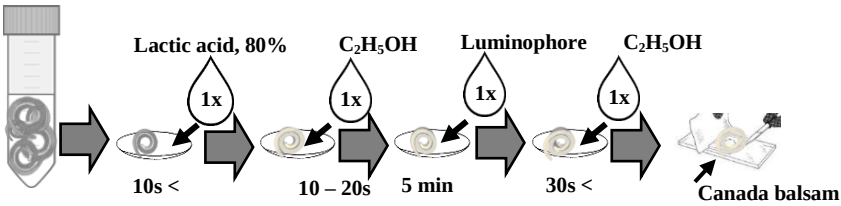


**Figure 3.** General staining process of Nematoda samples (Rubeniņa, 2022)

The developed staining protocol for *Trichinella* larvae using AZM luminophore was used to determine the sex of the larva. Gender determination was based on Kozek (1975) and Liu et al. (1991) studies which confirmed that the length of a male's rectum length can reach 63 μm and in females it is always half of that - 30 μm.

**Confocal laser scanning microscopy method (I, II, III, IV)**

After staining using benzanthrone luminophore, all parasites were studied using *Nikon Eclipse Ti-E* confocal laser scanning microscope (Nikon, Japan), which is configured with a high-speed multiphoton *A1R MP* confocal system and equipped with a digital *DS-U3* camera (Nikon, Japan). Three lasers were used to excite fluorescence: *Ch2* green laser λ= 488 nm with FITC filter (500–550 nm) (**I, II, III, IV**)); *Ch3* orange laser λ= 561 nm with TRICTfilter (570-620 nm) (**III, IV**) and *Ch4* red laser λ= 638 nm with Cy5 filter (662-737 nm) (**I**). All the obtained data was stored in .jpg and *ND2Image File* format, after the data was processed with *NIS Elements Advanced Research 3.2 64-bit* software (Nikon, Japan).

# RESULTS

**Fixation and storage of samples of Trematoda and Nematoda (I, II, III, IV)**

The effect of the chemical fixatives on samples was observed twice: during the preparation of the sample for storage and during confocal laser scanning microscopy (CLSM).

Chemical fixation of *P. fasciolaemorpha* (**I**) was carried out in six different fixatives. During fixation it was observed whether any morphological changes were occurring in the samples. Fixing Trematoda in Carnoy's solution led to an increase in size of the samples. The samples fixed in ethanol darkened slightly compared to the colour of the samples before fixing. The Bouin's solution stained all the samples yellow and the stain could not be rinsed off during further sample treatment. As a result, *P. fasciolaemorpha* fixed in the Bouin's solution influenced the quality of the results obtained in the CLSM study.

Freshwater trematodes (**III**) were stored in 96% ethanol, refrigerated at +4°C until the next examination. No morphological changes were observed in *D. spathaceum* (mtc.) and *D. subclaviatus* (ad.) that were stored for a week. Physical changes were observed in the samples of *P. confusus*, since during staining the sample decomposed and became too fragile. During CLSM, in the body of samples there were observed physical bursts – black spots.

During the study, *Trichinella* larvae (**II**) were used, the ones that were stored in 96.6% ethanol or the ones obtained from -20°C frozen muscle (**IV**). Immediately after isolation, *Trichinella* larvae were fixed in 5 different chemical fixatives. No physical damage was observed in the samples studied with light microscopy. In turn, examination of samples with CLSM revealed that older samples (3 years <) have degradation of the morphological structure. The studies confirmed that the chemical fixator has an effect on obtaining detailed CLSM data.

**Effective and rapid examination of samples of Trematoda and Nematoda (I, II, III, IV)**

Before the sampling and staining procedures, a control sample study was carried out. Autofluorescence studies were conducted with samples that were isolated from the host or frozen host muscle on a single day. The isolated samples were not chemically fixed and were not stained with benzanthrone luminophores (**I, III**). The unfixed and unstained samples were investigated by the CLSM method. As a result of autofluorescence, the overall body shape was illuminated in the trematode samples, where it was seen that the surface of the body was not smooth. In samples of *P. fasciolaemorpha*, it was possible to determine the position of the abdominal sucker and copulation organ. In *T. spiralis* and *T. britovi* autofluorescence experiments the used samples were fixed in chemical fixators (**II)**. The results confirmed that by using a 488 nm laser it is possible to achieve almost 10 times the autofluorescence attenuation signal compared to the absorption of the wavelength of 405 nm (**II, IV**).

The staining of *P. fasciolaemorpha* parasites was carried out in parallel with two luminophores in order to compare the obtained results. Study of the body of trematode (samples stained with AZPP) was possible already under x40 magnification (see Figure 4).

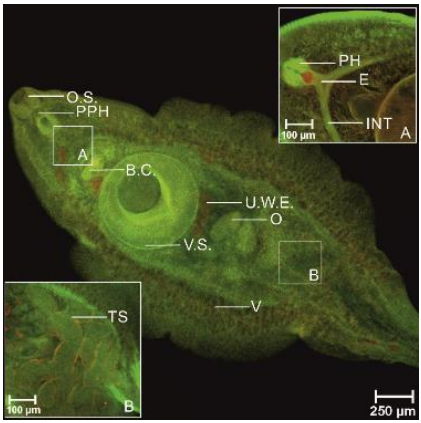


**Figure 4.** Adult *Parafasciolopsis faciolaemorpha* stained with AZPP dye, fixative AFA (single stack) (Rubeniņa, 2021). O.S.—oral sucker, PPH—pre-pharynx, PH—pharynx, E—esophagus, C—cirrus, V—vitellaria, TS—testes, O—ovary, INT—intestine, V.S.—ventral sucker, U.W.E.—uterus filled with eggs

Body surface structures, including body surface spikes and tegument, were observed. The spatial (dimensional) structure of the body was visualized. Simultaneously three layers of muscles were observed under the x100 magnification: circular, diagonal and longitudinal. Circular, longitudinal and radial muscle layers were observed in the suction cups of the mouth and abdomen. Parenchymal cells were clearly observed in the tail area of the trematode's body. The results confirmed that the most suitable luminophore and fixation combination for the study of *P. fasciolaemorpha* with CLSM is 70% ethanol or AFA solution and AZPP luminophore. The developed staining protocol using synthesized AZPP benzanthrone luminophore and CLSM is suitable for effective and rapid research of *P. fasciolaemorpha*, making the staining protocol less work and time consuming (**I**).

The microscopic examination results of *D. spathaceum* (mtc.) fixed in 96% ethanol, indicated the suitability of AM1, AM2, AM4, AM16 and P8 luminophores for Trematoda studies. Vivid luminiscence was observed in scolex, pseudo suction cups and primary excretory system (**III**).

CLSM results from studies with *D. subclaviatus* trematodes, fixed in Carnoy's solution, showed more detailed data by marking tiny, taxonomically important

skeletal elements (see Figure 5), but on the other hand, the samples did not show internal organs and structures, even when changing the scan settings (**III**).

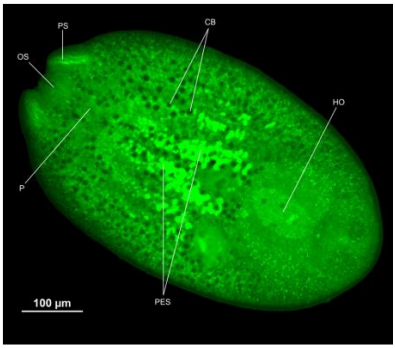


**Figure 5.** *Diplostomum spathaceum* mtc. stained with AM2 (Rubeniņa, 2018). OS - oral sucker; PS - pseudo suckers; P - pharynx; HO - holdfast organ; CB - calcareous bodies; PES - primary excretory system.

In samples of *P. confusus*, it was observed that the cuticle was covered with spikes. The digestive and reproductive system (see Figure 6) was observed (**III**).

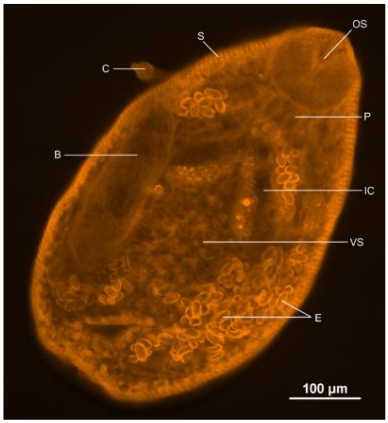


**Figure 6.** Adult *Prosotocus confusus* stained with AM1 (internal structure) (Rubeniņa, 2018).
OS - oral sucker; P - pharynx; IC - intestinal caeca; VS - ventral sucker; E - eggs; S - spines; B - bursa; C - cirrus.

For the study of *T. spiralis* and *T. britovi* larvae, P13 and AZM benzanthrone luminophores were synthesized. The use of benzanthrone luminophores and CLSM method made it possible to study in detail the body of the parasite,

including the morphology of organs and their arrangement in the body. The effectiveness of synthesized luminophores and developed staining protocols was tested by replacing luminophore with a commercially available dye. The results confirmed higher quality of data with the synthesised P13 and AZM benzanthrons, which showed differences in the structure of the cuticle between the two *Trichinella* species. "Pseudo-segmentation" was observed in the cuticle of *T. spiralis*, but no segmentation was observed in the cuticle of *T. britovi*, which led to the conclusion that it was smooth. The protocol developed by the AZM made it possible to accurately measure the length of the larva's rectum. Based on the length of the rectum, it is possible to determine the sex of the larva (see Figure 7). As a result, it was estimated that the average length of the rectum in females of *T. britovi* was 21.19 μm ± 2.45 SD, and *T. spiralis* 20.55 μm ± 1.48 SD. In males, the average length of the rectum was 41.08 μm ±4.26 SD and 46.08 μm ± 2.95 SD, respectively. Subsequently, in females, a bright fluorescence was observed in the uterus primordium.

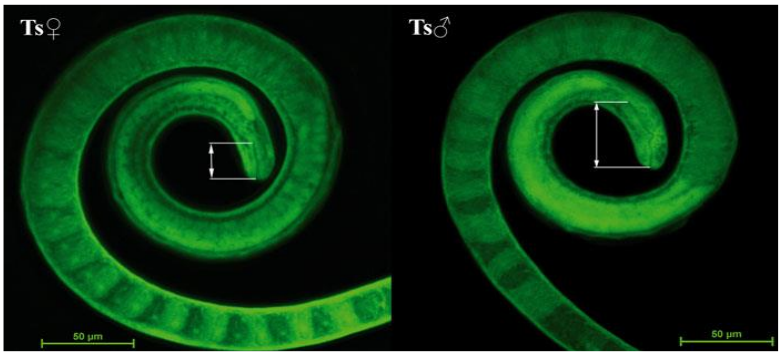


**Figure 7.** CLSM image of *Trichinella* larva female's and male's rectum (Rubeniņa, 2019). Ts♀ - *T. spiralis* female, Ts♂ - *T. spiralis* male. Rectum marked with ↔.

# DISCUSSION

Nowadays, more and more attention is paid to the staining of the biological objects with luminophores (Shivraj et al., 2018). Previous studies have confirmed that benzanthrone (3-N-2- [4- (2-feniletil) piperazin-1-il] acetamido benzanthrone (AZP5) is suitable for morphological studies of helminth fixed in 96% ethanol (Kirjusina et al., 2018). Kirilova et al. (2019) confirmed the use of benzanthrone luminophores for visualization of callus embryonic cells. In this context, new benzanthrone luminophores were synthesized that would visualize the cell membrane by localizing the colour in the membrane lipids (**I, II, III, IV**). When selecting the most suitable benzanthrone luminophores, it was found that the substituent at the C-3 position plays an important role in determining the efficiency of the luminophore in revealing the structural and environmental effects on the photophysical parameters. Synthesized luminophores have a wide range of polarity (Kapusta et al., 2003; Siddlingeshwar et al., 2011; Shivraj et al., 2018), confirming the use of luminophores to visualize various biological structures (Kalnina et al., 2007; Trusova et al., 2012; Ryzhova et al., 2016; Kirjusina et al., 2018; Kirilova et al., 2019).

During the use of benzanthrone luminophores, the photobleaching process must be considered, because of which the quality of the test sample decreases each time it is exposed to a laser (Han et al., 2021). In studies (**I, II, III, IV**) it has been confirmed that it is recommended to use laser wavelengths in descending order to obtain detailed results. The photobleaching process confirmed that the samples stained with benzanthrone luminophores and prepared for CLSM were suitable for rapid and efficient study of parasite species.

The hypothesis that a certain staining protocol using a specific benzanthrone luminophore and confocal laser scanning microscopy is suitable for efficient and rapid research of various parasite species was confirmed.

**Trematoda (I, III)**

CLSM is widely used in the study of the morphological and physiological structure of various species, especially for fixed trematode samples. (Jurberg et al., 2008; Borges et al., 2017). The experimental results of the study (**I**) showed that using a 488 nm (500-655 nm filter) laser excitation, it was possible to achieve a 23x lower autofluorescence signal compared to a 405 nm (425-580nm filter) wavelength excitation. When evaluating autofluorescence, different ROIs were selected, and the selected ROIs were compared with the background ROI. Based on the obtained data, 488 nm laser with FITC filter (500-550 nm) and 638 nm laser with Cy5 filter (662-737nm) were the most suitable lasers for suppression of unwanted autofluorescence. In the study with freshwater trematodes (**III**), a laser with a wavelength of 405 nm was not used because it induces autofluorescence of the samples. The benzanthrone luminophore synthesized in the study showed fluorescence in the red spectral region, however, the benzanthrone-stained samples showed a shorter

fluorescence shift in the shorter wave region (**I, III**). Possibly, due to more hydrophobic conditions (higher lipids, dehydration with ethanol). Adjacent luminescence can be caused by a chemical fixative. For example, with a mixture of formalin-containing chemical fixatives, more intense cell luminescence in the yellow-green region of the spectrum may be observed (Alfano et al., 1984).

The presence of excess water in the cell structures does not allow adequate transparency of the sample. Shigin (1996) performed a dehydration step by staining trematodes for light microscopy, and the results of the study (**I, III**) also confirmed the importance of dehydration in the development of the new protocol. The results of the study confirmed that for parasites with a thin-shelled structure, e.g., Diplostomatidae (**III**), there is no need for an additional transparency step using 100% xylene, as absolute xylene can deform the structure of more sensitive samples. During the study (**I**), it was observed that the thickness of the trematode determines how long the sample should be kept in the luminophore and how long in 100% xylene. The observations were included in the development of the staining protocol, which states that trematodes isolated from homothermic host should be kept in the luminophore five minutes longer compared to trematodes isolated from poikilothermic hosts. *P. fasciolaemorpha* is kept in ethanol/xylene solution for 8-10 min, then in 100% xylene for 30s - 3 min, such variation in duration is based on different thicknesses of parasite samples.

Trematodes are characterized by the structure of the body wall muscles, which consists of three layers: annular, transverse and diagonal (Ginetsinskaya, 1988; Galaktionov & Dobrovolskij, 2003). In the used research material (**I, III**) all three most characteristic layers of Trematoda body wall muscles were observed. Relatively complex staining methods are used to study muscle layers, such as the preparation of a sample by Krupenko (2014) for confocal laser scanning microscopy takes two days. In today's world, two days is too long, so opportunities were sought to develop a staining protocol that would take less time and man-hours, however, would provide detailed results. With the staining protocols developed in the study, the results can be obtained within the first two hours (**I, II, III, IV**). In the beginning of the first attempts to stain freshwater trematodes, the results did not show all expected muscle structure in the body, except for muscular throat, mouth, and abdomen suckers (**III**). In a study with *P. fasciolaemorpha* (**I**), a more detailed body muscle structure was observed by visualizing the layers of the annular, diagonal, and transverse muscles. The results obtained in studies (**I, III**) confirmed that the synthesized benzanthrone luminophores and the developed staining protocols gave similar results as when staining samples with fluorescein isothiocyanate or tetramethyl rhodamine B isothiocyanate-conjugate that directly stains actin (Terentina et al., 2020). The results of the study (**I**) confirmed the presence of spikes on the body surface, as confirmed by other researchers as well. (Rankin, 1939; Belopolskaya, 1963;

Davies, 1979; Saville et al., 1997; Pina et al., 2011; Krupenko & Dobrovolskij, 2018). With CLSM, it was observed that the anterior part of the body is more densely covered with spikes than the lower part of the body (**I, III**). Although Krupenko & Dobrovolskij (2018) concluded that the form of spikes, the number of teeth, etc. cannot be determined just by the CLSM method alone, the results of this study (**I**) confirmed the opposite. The AZPP luminophore and the CLSM method provided information on the size of the spikes, the number of teeth and their shape.

The results of study (**I**) confirmed the presence of all three characteristic muscle layers in the oral sucker: radial, equatorial (= circular) and meridional (= striated). It was concluded that the equatorial muscle fibers are more densely located directly in the copulation organ and in the copulation organ sac, compared to *Fasciola hepatica* (Mair et al., 1998). Overall, the results of the study (**I, III**) confirmed the general morphology of the body of the trematode according to Scrjabin (1949).

Glycogen reserves were observed in the parenchyma cells below the tegument, most notably in the vitellaria. Scattered glycogen reserves were observed in the attachment organs. The brightest fluorescence was observed in parasite eggs, which consist mainly of glycogen and lipids (**I**). Glycogen reserves and lipids serve as a source of energy, a regulator of cell activity, and are used to form biological membranes (Swiderski et al., 2019). The staining protocols developed in the study (**I, III**) were not intended for staining of any specific organ system. As a result, no parts of the nervous system were observed when staining the trematodes with the synthesized AZPP, AM323, AM1, AM2, AM4, AM16, and P8 benzanthrone luminophore. Benzanthrone luminophores with three different functional groups were used in the studies. The choice of luminophores was based on studies of Kapusta et al. (2003), Siddlingeshwar et al. (2011), Shivraj et al. (2018) and Tarabara et al. (2021) in which it was concluded that the functional group determines the efficiency of the luminophores, thus allowing the possibility that the results of the study could be influenced by the functional groups of benzanthrone. Overall, the results obtained (**I, II, III, IV**) confirmed the role of the luminophore functional group in obtaining qualitative data. The most efficient research of parasites was performed with benzanthrones having aminoamido groups (AZPP (**I**) and AZM (**II**)).

**Nematoda (II, IV)**

In studies of the genus *Trichinella*, CLSM has been mainly used to study excretory and secretory antigens (Li et al., 1999), immune responses (Bai et al., 2012), or the interaction of *T. spiralis* in muscle cells *in vitro* (Bai et al., 2011) and used in various studies evaluating pathological and / or therapeutic effects (Li et al., 1999; Masetti et al., 2004; Hao et al., 2014). The parasitic species *T.*

*spiralis* and *T. britovi* are the causative agents of trichinelosis, which is dangerous to humans and animals (Rozycki et al., 2022; Tso et al., 2022).
Usually, the range of excitation of autofluorescence is approximately 400 nm, and 488 nm absorption was chosen in the study (**II**) to suppress the autofluorescence signal relative to the fluorescence signal of the marker (Schnell et al., 1999; Neumann & Gabel, 2002). Absorption at 488 nm gave the best fluorescence / autofluorescence signal proportion (**II, IV**).
The data quality of the studied samples is influenced by the storage conditions of the studied samples. *Trichinella* larvae, like Trematoda samples (**I, III**), were fixed in several chemical fixators and it was confirmed that the chemical fixative has an effect on the result. The study (**II**) confirmed that the most appropriate chemical fixative for the effective study of *Trichinella* larvae is the Bouin's solution. The staining protocol developed in the study is suitable for larval samples isolated from animal muscles (**II**). The developed protocols are rapid and simple (**II, IV**) compared to the protocols developed by manufacturers, where sample preparation consists of multiple steps where one step can take up to several hours (Stewart et al., 2003a, b, c; Davila et al., 2010).
One of the signs that can determine the sex of *Trichinella* larvae is the length of the rectum. In males, the average length of the rectum is 40 μm to 50 μm, while in females it is almost half as size -17 μm to 35 μm. During the study, a staining protocol (**II**) was developed that is suitable for determination of the sex of the larvae by measuring the length of the rectum. The results of the study showed that the rectum length in T. *britovi* males is 41.08 ± 4.26 μm SD and T. *spiralis* - 46.08 ± 2.95 μm SD; for females 21.19 ± 2.45 μm SD and 20.55 ± 1.48 μm SD, respectively. The data obtained from the study are consistent with data from other studies and confirm that the rectum length of males is twice as long as that of females (Kozek 1975; Liu et al., 1991; Pozio & La Rosa, 2010).
The results of the study confirmed the typical body of the Nematoda (**II, IV**): a narrower anterior part and a wider posterior part. The larval cuticle consists of three or more outer layers formed of collagen and other components (Lichtenfels et al., 1983). The obtained results showed a high fluorescence signal in the larval cuticle (**II, IV**). The cause of the high fluorescence signal in the cuticle may be the accumulation of lipids in the epicuticle of *Trichinella* larvae (Gounaris et al., 1996). In general, the body of trichinella is covered by a ribbed cuticle (Hetherington, 1924), and during this study it was observed that there are differences between the cuticle of *T. spiralis* and *T. britovi* larvae. A corrugation, the so-called “pseudo-segmentation”, was observed in *T. spiralis* larvae, while no transverse lines or corrugation were observed in the cuticle of *T. britovi* (**II**). The muscle larvae have sufficiently developed organs (McVay et al., 1998). Studies have shown that the larval muscle has both a digestive and a reproductive organ system (**II, IV**). Stichosome and stychocytes were observed in both frozen samples stained with P8 (**IV**) and freshly isolated samples

stained with AZM (**II**). A digestive track consisting of a monolayer of epithelium with a basement membrane on the basal side was observed when staining samples with AZM (**II**). Scientific studies have confirmed that there are four types of epithelia in the digestive track of the *Trichinella*, and some of the epithelial cells are myoepithelium, which provides digestive track peristalsis (Takahashi, 2021). In studies (**II, IV**), different epithelial cells were not observed, but there is a possibility that they could be observed by optimizing the staining protocol. AZM-stained specimens showed both middle intestine and genital primordium (**II**), while P8 showed only genital primordium (**IV**). The entire digestive system (**II**) was stained with AZM. Another feature to separate a male from a female larva is the amount of glycogen stored in the genital primordium (Takahashi et al., 1987). In females, the glycogen amount is higher, so staining samples with benzanthrone luminophore will result in more intense fluorescence at higher glycogen levels. The evaluation of the results concluded that glycogen reserves in female larvae are better visualized if stained with P8 (**IV**).

In the AZM luminophore study, a nerve ring was observed, but no more detailed structures were observed (**II**). Parts of the nervous system were not visualized with the P8 luminophore (**IV**).

A total of nine benzanthrone luminophores were synthesized for effective and rapid study of *P. fasciolaemorpha* (ad.) (AM323, AZPP), *D. spathaceum* (mtc.), *D. subclaviatus* (ad.) and *P. confusus* (ad.) (AM1, AM2, AM4, AM16, P8), *T. spiralis* and *T. britovi* (AZM, P13). Summarizing all results, it should be concluded that the most qualitative data for both Trematoda and Nematoda specimens were obtained with benzanthrone containing amidoamido functional group. Specific staining protocols were developed for the efficient and rapid study of parasite species using CLSM with detailed results within two hours. During the protocol development stage, it was noticed that staining step for trematode samples is determined by host (poikilotherm or homotherm) from which the trematode has been isolated. Staining protocols can be used for samples fixed seven different chemical fixators, as well as for three different types of *Trichinella* larval parasites: larvae isolated from animal muscles and fixed in the same day, larvae stored in 96% ethanol and larvae in frozen animal muscles. By using specific benzanthrone luminophores and certain staining protocols, it is possible to determine the differences in the cuticle structure of *T. spiralis* and *T. britovi* and to accurately determine the sex of both *Trichinella* larvae.

## CONCLUSIONS

1. A total of nine synthesized benzanthrone luminophores were approbated for detailed and rapid investigation of Trematoda class and Nematoda phylum parasite species (**I, II, III, IV**).
2. For the study of *P. fasciolaemorpha* (ad.) (I), *D. spathaceum* (mtc.), *D. subclaviatus* (ad.), *P. confusus* (ad.) (**III**) parasite species fixed in 70% and 96% in ethanol, in AFA, Carnoy's and Bouin's solution and in 10% neutral buffered formalin; efficient and rapid staining protocols using the AM323, AZPP, AM1, AM2, AM4, AM16, and P8 benzanthrone luminophores were developed for detailed study. For the detailed study of the anatomical and muscular arrangement of *P. fasciolaemorpha* parasite samples with AZPP luminophore, samples fixed in 70% ethanol or AFA solution are the most suitable: 70% ethanol and AZPP luminophore for external structures and muscle layers; AFA solution and AZPP luminophore for internal structure studies. The Bouin's fixative is not suitable for fixation of *P. fasciolaemorpha* samples when analyzed by CLSM (**I**). The AM1, AM2, AM4, AM16, and P8 luminophores allow detailed visualization of the digestive and reproductive systems of *D. spathaceum* (mtc.) fixed in 96% ethanol, *D. subclaviatus* (ad.) fixed in Carnoy's solution, *P. confu*sus (ad.) fixed in AFA in solution (**III**). It was concluded that staining trematodes isolated from the poikilotherm host requires less time than staining trematodes isolated from the homotherm host.
3. For study of *T. spiralis* and *T. britovi* parasite species which were fixed in 70% and 96.6% ethanol; efficient and rapid staining protocols using AZM and P13 benzanthrone luminophores were developed in AFA, Carnoy's and Bouin's solution. It was concluded that the synthesized benzanthrone luminophore AZM is suitable for trichinella research isolated from animal muscles and fixed in various chemical fixatives. The developed staining protocol is suitable for determining the sex of *T. spiralis* and *T. britovi* larvae by measuring the length of the rectum length (**I**). Staining protocol with P13 was developed for *T. britovi* larvae stored in 96.6% ethanol or larvae frozen within animal muscles (**IV**).
4. Certain staining protocols using specific benzanthrone luminophores have been developed for the study of Trematoda class and Nematoda phylum parasite species. The most suitable chemical fixator and benzanthrone luminophore complexes were found for the study of samples using CLSM: *P. fasciolaemorpha* (ad.) fixed in 70% ethanol or AFA solution and AZPP (**I**); *T. spiralis* and *T. britovi* fixed in Bouin's solution and AZM (**II**); *D. spathaceum* (mtc.) fixed in 96% ethanol, *D. subclaviatus* (ad.) fixed Carnoy's solution, *P. confusus* (ad.) fixed in AFA solution and AM1, AM2, AM4, AM16 and P8 (**III**); *T. britovi* fixed in 96.6% ethanol for up to three years or frozen larvae in animal muscles for up to two years and P13 (**IV**).

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