European Union Reference Laboratory for Fish and Crustacean Diseases
NATIONAL INSTITUTE OF AQUATIC RESOURCES, TECHNICAL UNIVERSITY OF DENMARK

Report of the

12th Annual Workshop of the National Reference Laboratories for Crustacean Diseases

Kgs. Lyngby, Denmark

June 2nd 2021

Organized by the European Union Reference Laboratory for Fish and Crustacean Diseases, National Institute of Aquatic Resources, Technical University of Denmark, Kgs. Lyngby

Contents

Introduction and short summary

The 12th Annual Workshop of the National Reference Laboratories for Crustacean Diseases was held virtually on 2nd of June 2021. Because of the Covid-19 pandemic and the resulting limitations on travelling to and from Denmark, the workshop was held virtually using the Zoom platform.

The virtual organization of the meeting has allowed a significant expansion on the number of participants attending the workshop as well as for people in oversea countries to participate. In total, 77 participants from 40 countries attended the workshop.

The workshop was held back to back with the 25th Annual Workshop for National Reference Laboratories for Fish Diseases. There were two sessions with a total of 10 presentations.

On May 31st a special session dedicated only to the staff of NRLs in Europe was held to present the new Animal Health Law which was implemented by April 21st, 2021, and includes implications for Aquatic animal health.

The workshop was opened with "Welcome and announcements" by Head of the EURL for Crustacean Diseases, Niels Jørgen Olesen. The first session had the title "Update on important crustacean diseases and their control", and the first speaker was Hans Nauwynck from Ghent University who talked about the role of the shrimp nephrocomplex in molting and disease. This was followed by a talk by Peter Bossier, also from Ghent University, about phenotype switching in Vibrio bacteria. Charlotte Davies from Swansea University then presented an investigation of two new Haplosporidia species infecting shore crabs, which was followed by a talk by Jamie Bojko from Teesside University on invasive Crustacea and travelling pathogens. After a short break, where participants were invited to informal meetings in breakout rooms, the session continued with a presentation of a project about *Coxiella cheraxi* infection in *Cherax quadricarinatus* by Tobia Pretto from Italian health authority and research organization for animal health and food safety. Anna Aspan from the National Veterinary Institute of Sweden then presented a study to develop a new qPCR assay to detect *Thelohania contejeani* in tissue samples of nobel crayfish. The session ended with a talk by Fiona Swords from The Marine Institute Ireland on the second Irish National Crayfish Plague Surveillance Programme.

Session II had the title "Update from the EURL for fish diseases" and started with EURL coordinator Morten Schiøtt giving two talks, the first on the disease and surveillance situation of crustacean diseases in EU countries, and the second on the interlaboratory proficiency tests for crustacean diseases in 2021. The final talk was given by Niels Jørgen Olesen, presenting the EURL activities in year 2020 and proposals for the EURL work plan for 2021 and 2022.

Niccolò Vendramin and Morten Schiøtt from DTU Aqua took minutes from the meeting, and Morten Schiøtt assembled the report.

We would once again like to thank all the presenters for their great contribution, without them the meeting would not have been a success. The workshop and meeting was organized by a team consisting of Morten Schiøtt, Niccoló Vendramin and Niels Jørgen Olesen, with the help from the rest of the fish and crustacean disease section at the National Institute of Aquatic Resources, DTU

AQUA. The meeting next year is tentatively planned to be held at beginning of June 2022, hopefully in a face to face meeting at DTU Aqua. More details will follow.

We wish to thank all of you for participating and we are looking forward to seeing you next year.

Niels Jørgen Olesen and Morten Schiøtt

Programme

Wednesday June 2nd
Annual Workshop of the National Reference Laboratories for Crustacean Diseases

9.30 – 9:40	Welcome and announcement *Morten Schiøtt and Niels Jørgen Olesen*
SESSION I:	**Update on important crustacean diseases and their control**
	Chair: Lone Madsen and minutes: Morten Schiøtt
09:40 – 10:00	The nephrocomplex (formerly known as 'antennal gland'), the golden gate for pathogens and central engine for the molting process *Hans Nauwynck*
10:00 – 10:20	Mitigation of AHPND based on phenotype switching in Vibrio parahaemolyticus *Peter Bossier*
10:20 – 10:40	Investigating disease dynamics in shore crabs, *Carcinus maenas*, including two new Haplosporidia species *Charlotte E Davies*
10:40 – 11:00	Invasive Crustacea and Travelling Pathogens *Jamie Bojko*
11:00 – 11:20	***Coffee break***
	Chair: Morten Schiøtt and minutes Niccoló Vendramin
11:20 – 11:40	*Coxiella cheraxi* infection in *Cherax quadricarinatus* imported from Australia *Tobia Pretto*
11:40 – 12:00	qPCR detection of *Thelohania contejeani* (and *Aphanomyces astaci* & WSSV) from tissue samples of nobel crayfish *Anna Aspan*
12:00 – 12:20	Preliminary results from the second Irish National Crayfish Plague Surveillance Programme *Fiona Swords*
SESSION II:	**Update from the EURL for crustacean diseases**
12:20 – 12:40	Surveillance and diagnostics of crustacean diseases in Europe *Morten Schiøtt*
12:40 – 12:50	EURL proficiency test for crustacean disease 2021 *Morten Schiøtt*

12:50 – 13:10	EURL Work done in 2020, plan for 2021 and ideas and plans for 2022 *Niels Jørgen Olesen* Next meeting and end of 12th Annual Workshop *Niels Jørgen Olesen*

SESSION I: Update on important crustacean diseases and their control

Chair: Lone Madsen

The nephrocomplex (formerly known as 'antennal gland'), the golden gate for pathogens and central engine for the molting

H.J. Nauwynck

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Abstract

Viruses, such as white spot syndrome virus, and bacteria, such as Vibrio species, wreak havoc in shrimp aquaculture. As the main portal of entry for various pathogens in shrimp remain unclear, infectious diseases are difficult to prevent and control. Because the cuticle is a strong pathogen barrier, regions that lack cuticular lining, such as the shrimp's excretory organ, "the antennal gland", are major candidate entry-portals. The antennal gland, up till now morphologically underexplored, was studied using several imaging techniques. Using histology-based 3D-technology, we demonstrated that the antennal gland resembles a kidney, connected to a urinary bladder with a nephropore (exit opening) and a complex of diverticula, spread throughout the cephalothorax. Micro Magnetic Resonance Imaging of live shrimp not only confirmed the histology-based model, but also indicated that the filling of the diverticula is linked to the molting cycle and possibly involved therein. Based on the hemolymph filtration function and attached diverticle complex, we propose to rename the antennal gland as the "nephrocomplex". By an intrabladder inoculation, we showed high susceptibility of this nephrocomplex to both white spot syndrome virus and Vibrio infection compared to peroral inoculation. An induced drop in salinity allowed the virus to enter the nephrocomplex in a natural way and caused a general infection followed by death; fluorescent beads were used to demonstrate that particles may indeed enter through the nephropore. These findings pave the way for an oriented disease control in shrimp.

Questions and comments:

Q: Will a drop in salinity, e.g. after heavy rain fall, be a trigger for virus uptake through the nephrocomplex?

A: Yes, it is important to keep temperature and salinity constant. Shrimp pee on their food, so infecting shrimp using infected carcasses is an effective method of doing infection experiments.

Q: Does the shrimp also shed viruses this way?

A: Probably, but more importantly through eating dead infected shrimp, virus particles will be released to the water and taken up via the nephrocomplex.

Mitigation of AHPND based on phenotype switching in Vibrio parahaemolyticus

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Abstract

AHPND is a disease caused mainly by Vibrio parahaemolyticus. The virulent strains harbor a plasmid encoding for the PirA/B toxin genes. AHPND strains display 2 phenotypic stages. In the planktonic form pirA/B toxins are produces and secreted. In the auto-flocculating form, toxins are not produced, but rather an alkaline phosphatase is produced and secreted. Expression of the toxin genes or the alkaline phospha PhoX gene can be used as marker for their phenotypic status. This study aimed at developing AHPND mitigation strategies that profit from the phenotype switching. First a biofloc system was verified, to describe the underlying mechanism behind the AHPND protective effect of a biofloc system in *Litopenaeus vannamei*. First, the results confirmed that a biofloc system maintained at a C/N ratio of 15, improves the water quality and contributes to the nutrition of cultured animals as bioflocs might serve as an additional protein source. Secondly, the study demonstrated that the biofloc system enhances the survival of *L. vannamei* upon challenge with a *V. parahaemolyticus* AHPND strain. Remarkably, the results highlight that in the biofloc system, AHPND-causing *V. parahaemolyticus* possibly switch from virulent planktonic phenotype, producing AHPND toxins, to a non-virulent biofilm phenotype (not producing APHND toxins), as demonstrated by a decreased transcription of flagella-related motility genes (*flaA*, *CheR*, and *fliS*), Pir toxin ($PirB^{VP}$), and AHPND plasmid genes (*ORF14*). In contrast an increased expression of the phenotype switching marker *AlkPhoX* gene was observed in both *in vitro* (in the biofloc) and *in vivo* (in the stomach of biofloc-based shrimp) conditions. Taken together, results suggest that bioflocs steer phenotype switching, contributing to the decreased virulence of *V. parahaemolyticus* AHPND strain towards shrimp postlarvae. In addition it was found that bamboo powder as a substratum is also inducing a phenotypic switch. This information opens the possibility to combat AHPND not only by trying to eliminate the AHPND-causing *V. parahaemolyticus* from the system but rather to steer the system allowing for a phenotypic switch of *V. parahaemolyticus*

Questions and comments:

Q: Can lowering the flow induce low virulent strain?

A: Shear stress may induce virulent strain

Q: Will RAS systems increase low virulent biofilm?

A: Sometimes the bacterium is present but not causing disease, maybe the bacterium can stay hidden in biofilms, and then change phenotype for unknown reasons.

Q: Does any of you have experience in extracting cortisol from shrimp and/or their environment (feces, water)?

A: We have been doing that in Artemia helped by the company https://stresschron.eu/contact.

Investigating disease dynamics in shore crabs, *Carcinus maenas*, including two new Haplosporidia species

Charlotte E. Davies[1], Jessica Thomas[1], Sophie Malkin[1], Frederico Batista[2], Christopher J. Coates[1] and Andrew F. Rowley[1]

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Abstract

The green, or common shore crab, *Carcinus maenas*, although native to Britain and Ireland, has been introduced to the USA, Sri Lanka, Red Sea, Madagascar, South Africa and Australia. This species hosts a range of micro- and macro-parasites and due to its introduction to a wide range of areas and sharing of habitats with species of commercial importance, it is an important species in which to monitor disease.

Crabs (n=50/location) and water (2L/location) were sampled monthly from two distinct locations; a closed Dock and an intertidal Pier, over 12 months. Molecular screening of both crab DNA and water eDNA, in addition to histological screening of crab gills and hepatopancreas for several infections took place.

Overall, 13.6% of crabs were positive for the most common disease, *Hematodinium* spp., from PCR analyses (14.4% Dock and 12.8% Pier location) with significant patterns according to season, sex and size. However, both the significant patterns as well as the diversity of co-infections differed according to location. *Hematodinium* spp. were found in eDNA of just the Pier location. Notable additional infections detected included a novel mycosis and two new Haplosporidian species, which again, were only found in the Pier location.

Due to the increasingly wide range of *C. maenas*, as well as the site-specific differences seen, it is imperative to further understand the ecology of these diseases in terms of environment and connectivity alongside traditional diagnostics.

Questions and comments:

Q: Have you looked for specific Vibrio species?

A: Found Vibrio in every single sample, but only a very short fragment was amplified, so we were not able to identify the species.

Invasive Crustacea and Travelling Pathogens

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Abstract

Invasive non-native species (INNS) pose a risk as vectors of parasitic organisms (Invasive Parasites). Introducing invasive parasites can result in ecological disturbances, leading to biodiversity loss and native species illness/mortality, but occasionally can control INNS limiting their impact. Risks to human health and the economy are also associated with INNS and invasive parasites; however, we understand little about the diversity of symbiotic organisms co-invading alongside INNS. This lack of clarity is an important aspect of the 'One Health' prerogative, which aims to bridge the gap between human, wildlife, and ecosystem health.

To explore known symbiont diversity, we use the Crustacea (n=323) as an example group out of 1054 aquatic invertebrates classed as INNS across databases. We compile literature (year range 1800-2017) on all identified symbionts of INN crustaceans (crab, lobster, crayfish, shrimp, amphipod, isopod, copepod, barnacle, other). Our search indicated that 31.2% of INN crustaceans were known to hold at least one symbiont, whereby the remaining 68.8% had no documented symbionts. The symbiont list mostly consisted of co-invasive helminths (27% of the known diversity) and protists (23% of the known diversity), followed by bacteria (12%) and microsporidians (12%). *Carcinus maenas*, the globally invasive and extremely well-studied green crab, harboured the greatest number of symbionts (n=72). Based on the number of co-invaders in *C. maenas*, we suggest that each INN crustacean may truly harbour a similar symbiont diversity and predict, for the Crustacea group, that >23,000 symbionts may have the potential to be co-invasive.

We reveal that few studies provide truly empirical data that connect biodiversity loss with invasive parasites and suggest that dedicated studies on available systems will help to provide vital case studies. Despite the lack of empirical data, co-invasive parasites of invasive invertebrates appear capable of lowering local biodiversity, especially by causing behavioural change and mortality in native species. Alternatively, several invasive parasites appear to protect ecosystems by controlling the impact and population size of their invasive host.

The consequence of limited parasite screening of INNS, in addition to the impacts invasive parasites impart on local ecologies, are explored throughout the review. We conclude in strong support of the 'One Health' prerogative and further identify a need to better explore disease in invasion systems, many of which are accountable for economic, human health and ecological diversity impacts.

Questions and comments:

Q: Will climate change have an effect on invasions?

A: It is expected that increasing temperature will have an effect on host species, but it is still unknown how it will affect the parasites. High temperature will also be stressful to crustaceans, which may make them more vulnerable to parasites.

An outbreak of crayfish rickettsiosis caused by *Coxiella cheraxi* in redclaw crayfish (*Cherax quadricarinatus*) imported to Israel from Australia

Tobia Pretto[1], Eleonora Fiocchi[1], Andrea Basso[1], Jane Budai[1], Francesco Montesi[1], Nadav Davidovich[2]

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Abstract

Redclaw crayfish rickettsiosis, caused by *Coxiella cheraxi*, was reported in the early 2000s, in Australian and Ecuadorean facilities. *Coxiella cheraxi* is a rod-shaped intracellular bacterium that cause mortality up to 80% in *Cherax quadricarinatus* and it is closely related to *Coxiella burnetii*, the agent of Q-fever, which affects ruminants worldwide. In this study, we describe an outbreak of *C. cheraxi* in a batch of redclaw crayfish imported to Israel from an Australian hatchery.

An increase in the mortality rate, observed 2 months after introduction in a quarantine facility, was investigated through histopathology, revealing infection by rickettsia-like organisms (RLO). After that, a more extensive sampling was conducted and affected specimens were fixed for histological, molecular and ultrastructural examination. Histology revealed severe and systemic cytoplasmatic RLO proliferation in haemocytes and fixed phagocytes. TEM evaluation of the haemocytes of the hepatopancreatic haemal spaces confirmed the presence of bacteria referable to the genus *Coxiella*, while molecular analysis retrieved 16S rRNA sequences completely homologus with *C. cheraxi* published sequence. *Coxiella cheraxi* infection caused a total loss of almost 99% of the crayfish population in 5 months.

qPCR detection of *Thelohania contejeani* (and *Aphanomyces astaci* & WSSv) from tissue samples of noble crayfish

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Abstract

Cotton-tail disease is caused by a microsporidium, *Thelohania contejeani*, which infects and multiply mainly in crayfish muscle tissues. Heavily infected tissue appear porcelain white, which has given the disease its common name. As over time the tissue is destroyed, the animals ability to move and feed is affected, and it finally dies. How the parasite is transmitted between crayfish is still unclear, probably directly from crayfish to crayfish (via cannibalism), perhaps also via spore-infected gear, bottom sediment and water. There is currenlty no cure or therapy available.

The incidence of cotton-tail disease in Sweden is concidered to be low in wild crayfish populations, but in some cases infection rates up to 30 percent have been reported. The parasite can be a population limiting factor in wild populations. Hence, restocking of noble crayfish with Thelohania infection is not allowed according to Swedish legislation. The County Administrative Board can refuse a restocking permit without prior health examination. Thus effective and sensivite diagnostics are warranted. At present histology examination is used, which is both time consuming and probably not very sensitive in detecting carrier animals.

In our health monitoring of crayfish, qPCR is used both for the detection of crayfish plauge and the white spot syndrome virus (WSSv). A qPCR for *T contejeani*, using the same DNA extracts, would be both cost and time efficient.

Challanges in developing a qPCR for *T. contejeani* are both the limited amount of sequence data available in eg GenBank, as well as the limited number of well charaterized positive tissue material for method valdiation. Also, different disease agents have different tissue tropism, and the choise of tissue material can affect the sensitivity of a diagnostic metod.

We present a qPCR that was shown to be specific for *T. contejeani* both bioinformatically and experimentally. There was no cross-reactivity with other crayfish pathogens such as *Ahpanomyces astaci*, WSSv, *Psorospermium* or with DNA from signal or noble crayfish.

In samples from diseased noble crayfish, sent to our lab in 2020, one batch out of five tested was found to be qPCR positive for *T. contejeani*, which also was confirmed by sequencing. Further evaluation of the method on clinical samples will be done during 2021.

As the availability of positive reference material is scares, we invited other labs to do further evaluation of the qPCR in collaboration with us.

Preliminary results from the second Irish National Crayfish Plague Surveillance Programme

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Abstract

Austropotamobius pallipes (White-clawed crayfish; WCC) is the only crayfish species indigenous to Ireland. While the Irish population of WCC is still considered the healthiest in Europe, the latest species assessment under Article 17 of the EU Directive on the Conservation of Habitats, Flora and Fauna (92/43/EEC), reports their future prospects and overall status as bad. The main threats to this endangered and protected species are habitat destruction, the introduction of non-native species and the risk posed by *Aphanomyces astaci* (*A.astaci*), the causative agent of crayfish plague.

Since 2015, several outbreaks of crayfish plague have decimated crayfish populations in some Irish river systems. In response to the spreading plague, an environmental DNA (eDNA) based National Crayfish Plague Surveillance Programme was established in 2018. Following the successful first 2-year cycle in 2018-2019, a new collaboration between the National Parks and Wildlife Service and the Marine Institute was established with a second 2-year surveillance programme beginning in July 2020. Throughout 2018-2019 608 water samples from 28 catchments were tested for the presence of *A.astaci*, *A.pallipes* and 8 non-native crayfish species using real-time PCR. Seven new catchments tested positive for *A.astaci* at at least one site, bringing the number of crayfish plague affected catchments in Ireland to 12. These results suggested a rapid spread of the crayfish plague both within and between catchments. Genotyping of crayfish plague-positive samples from mortality events suggests multiple possible introduction pathways are possible. The distribution of WCC populations from the 2018-2019 survey confirms the decline described in the Article 17 assessment.

The preliminary results from the first year of the 2020-2021 survey will be presented here. The programme's scope has been widened, and the methodology further developed, with efforts to assign a genotype identity to eDNA samples ongoing as we attempt to pinpoint the origin of the infection. This study aims to further understand and monitor the outbreaks of crayfish plague in Ireland, its vectors, and its impact on the native WCC and to aid the relevant bodies in developing disease control measures to protect the endangered WCC in Ireland.

SESSION II: Update from the EURL for crustacean diseases

Surveillance and diagnostics of crustacean diseases in Europe

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Abstract

As part of being the EURL for crustacean diseases, we see it as our obligation to collect and disseminate data on the disease situation for crustacean production in Europe. To that end we send out an inquiry to all European NRLs for crustacean diseases to:

1) Report the number of farms belonging to each health status category listed in council directive 2006/88.

2) Report any outbreaks in the country of EU listed crustacean diseases, as well as health problems related to other crustacean diseases.

3) Report the number of samples tested for OIE listed crustacean diseases and how many of these gave a positive result.

4) Describe the current status of crustacean aquaculture in the country, as well as the strategy used for surveillance of crustacean diseases.

Despite a short notice, 19 out of 26 NRLs have so far responded to our inquiry. 12 NRLs report to have crustacean farms in their country, with 8 countries having shrimp farms and the remaining mostly being small scale crayfish farms or lobster farms for restocking of natural populations. Only very few incidents of disease have been reported, and very few NRLs perform regular disease surveillance at the farms. All in all, crustacean farming is still in its infancy in Europe, but seems to be increasing.

2021 Inter-laboratory proficiency test for crustacean diseases

Morten Schiøtt, Teena Vendel Klinge and Niels Jørgen Olesen

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Abstract

In June-August 2021 an inter-laboratory proficiency test for White Spot Syndrome Virus (WSSV), and another inter-laboratory proficiency test for Taura Syndrome Virus (TSV) and Yellow Head Virus (YHV) is organised by the EURL for Fish and Crustacean Diseases. The test material for the WSSV test will consist of shrimp pleopods infected with WSSV or not. The participants are asked to identify the WSSV positive pleopods among five test samples. 25 laboratories in 18 EU and 2 EFTA member states have signed up for the test. The test material for the TSV/YHV test will consist of FTA cards incubated with tissue extracts of TSV infected shrimp, YHV-1 infected shrimp or non-infected shrimp. The participants are asked to identify the TSV and YHV positive samples among six test samples. 16 laboratories in 12 EU member states signed up for the test.

This presentation will briefly give some recommendations for how to analyse the samples included in the inter-laboratory proficiency test.

Questions and comments:

Q: I know we have dealt this topic on several times, but it would possible to organise a Histology PT for WSSV?

A: Maybe this will be possible at some point, but we need to improve our skills in this before it will be possible. Also it is uncertain if the EU will pay for this, given that histopathology is not part of the diagnostic procedure.

EURL Work done in 2020, plan for 2021 and ideas and plans for 2022

Niels Jørgen Olesen

EURL for Fish and Crustacean Diseases,

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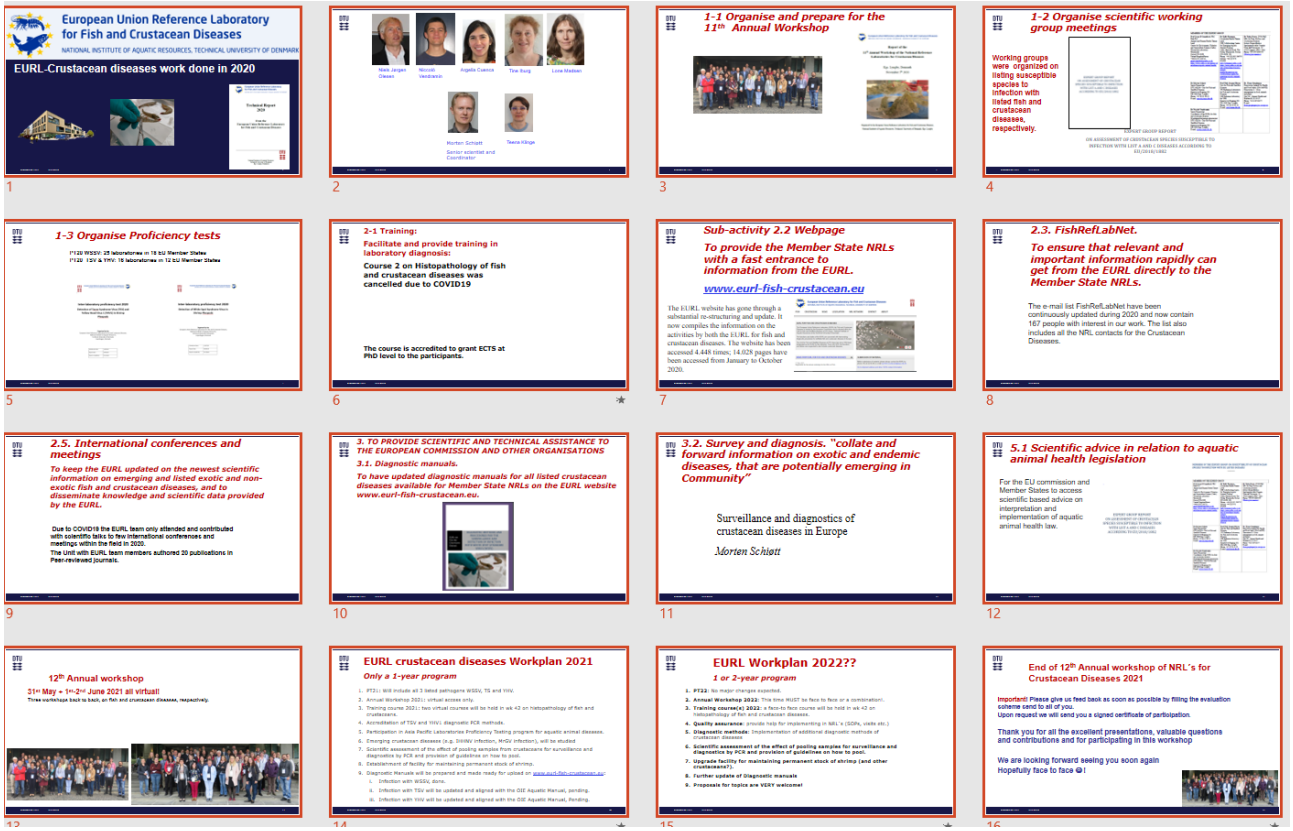
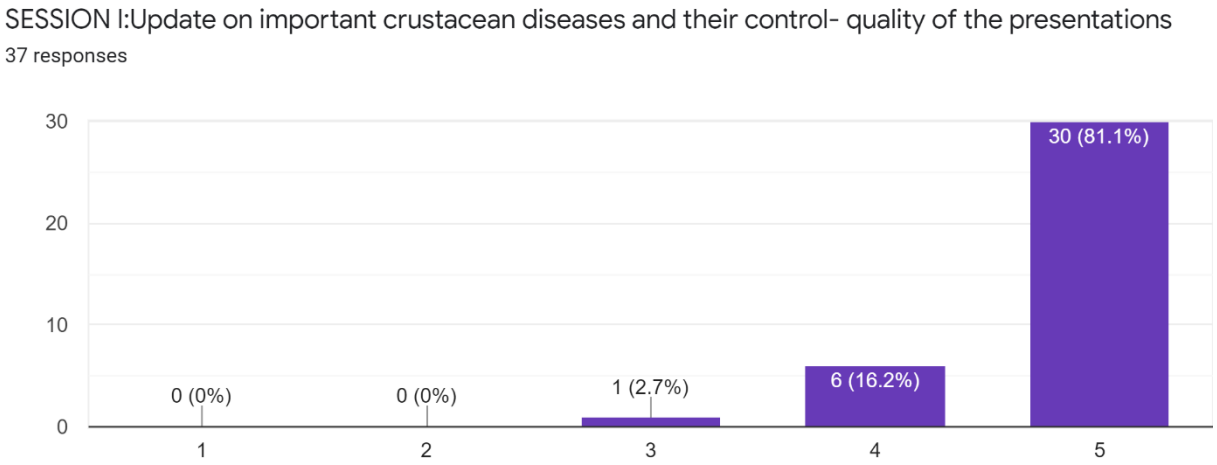
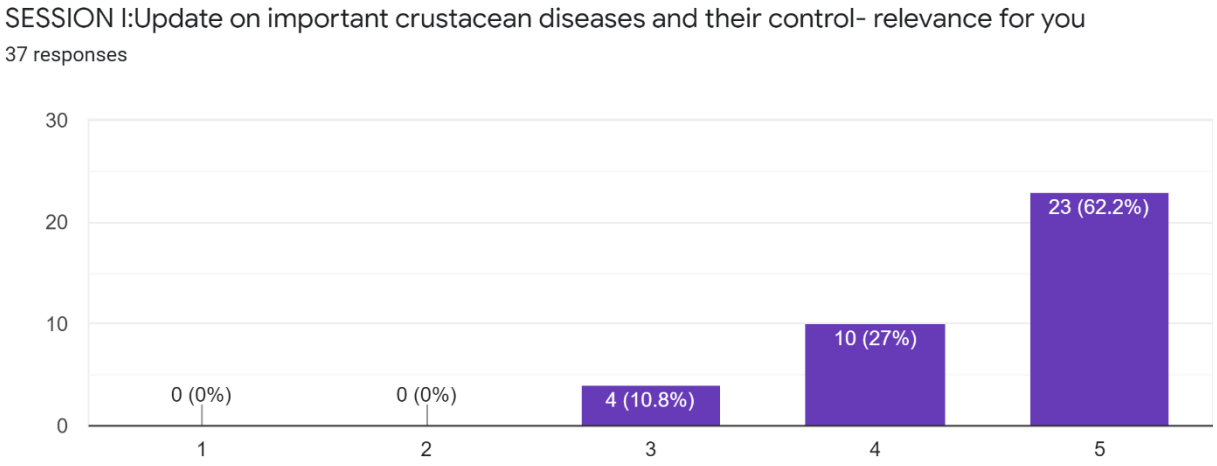
Abstract



Workshop evaluation

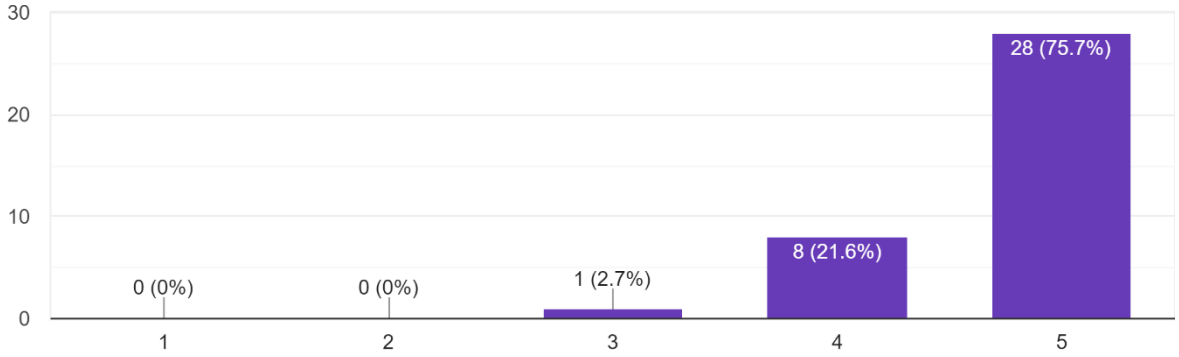
A questionnaire was delivered to the participants asking to evaluate various aspect of the workshop. An overview of the 37 questionnaires retrieved is shown below. Specific comments are going to be considered for the next annual workshop organization.





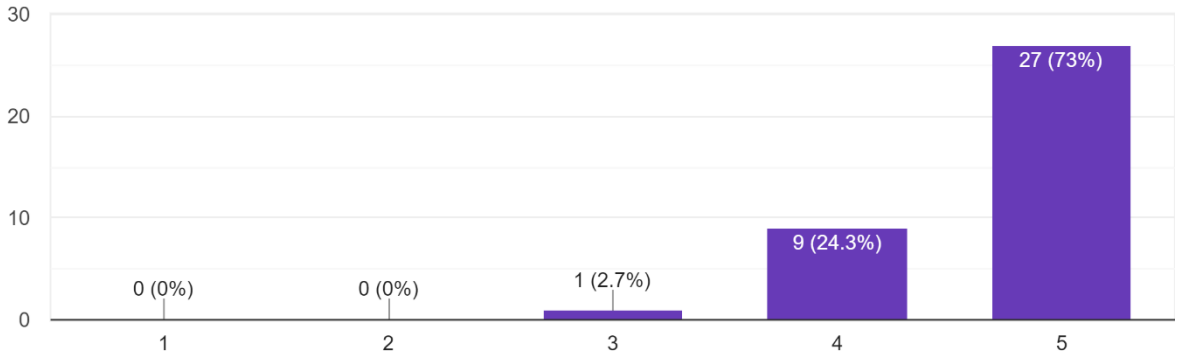
SESSION I:Update on important crustacean diseases and their control- overall score

37 responses



SESSION I:Update on important crustacean diseases and their control- increase of your knowledge

37 responses



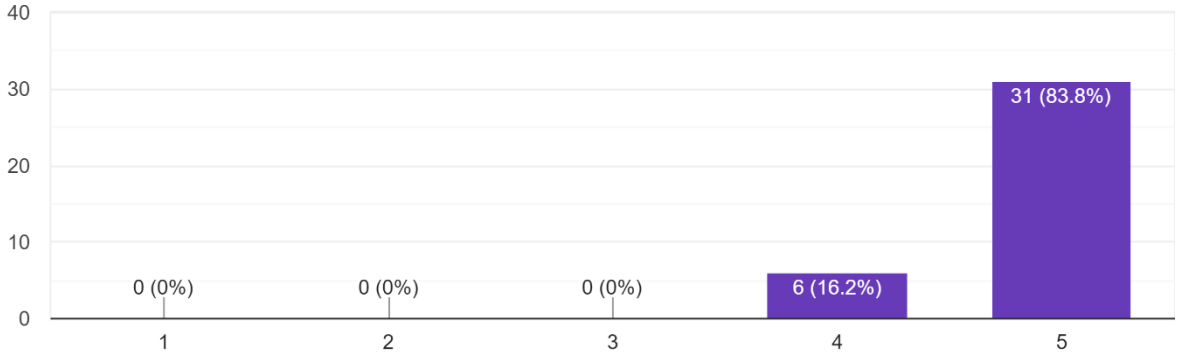
SESSION I:Update on important crustacean diseases and their control- comments, feedback, input

7 responses

Quality of the images in TEM and histology was excellent
Very useful. There were both general and very specific topics included
Thanks againa
nice and interesting presentation
excellent workshop
Excellent organization
Very interesting lectures, although presentation of Fiona too detailed regarding locations in Ireland, but in general of interest (eDNA)!

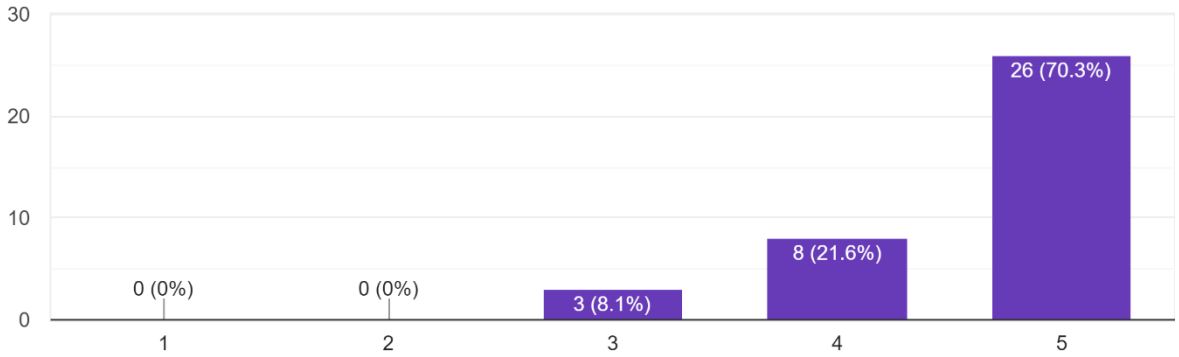
SESSION II:Update from the EURL for crustacean diseases- quality of the presentations

37 responses



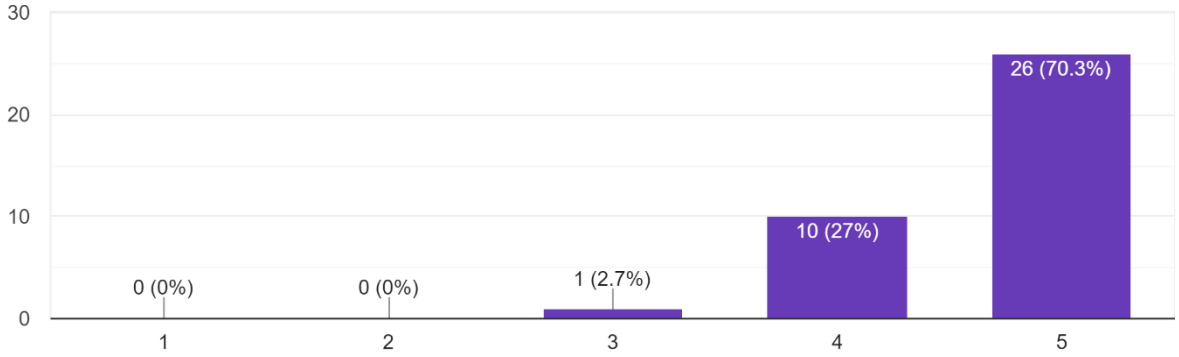
SESSION II:Update from the EURL for crustacean diseases- relevance for you

37 responses



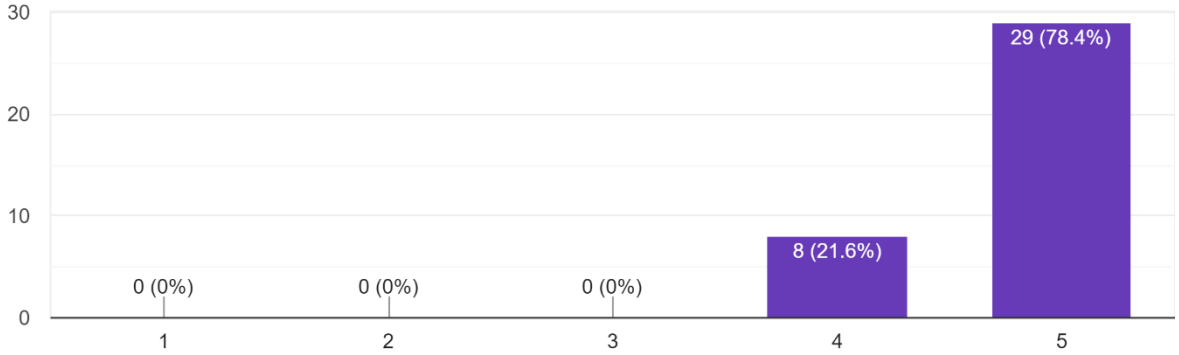
SESSION II:Update from the EURL for crustacean diseases- increase of your knowledge

37 responses



SESSION II:Update from the EURL for crustacean diseases- overall score

37 responses



SESSION II:Update from the EURL for crustacean diseases- comments, feedback, inputs.

8 responses

To hear about other diseases explained so clearly was very engaging. The speakers employed really good diagrams and the topics were interesting even if not immediately relevant to my job - it is always stimulating to hear others talk enthusiastically about their work. Wonderful workshop.
Lovely, informative presentation. Definitely increased my knowledge in crustacean research and advancements.
nice and interesting presentation
excellent workshop
Excellent presentations

The Surveillance data are for the first time collected. Next year we can see the dynamics. The ILPT announcement was very clear, important.
keep going!

Greetings and conclusions of the meeting

The tentative dates for the next meeting will be the 30th of May – 1st of June 2022. It will most likely be organized as a physical meeting in Lyngby, Denmark, possibly combined with an option for virtual participation. Thanks a lot to the people arranging the meeting as well as those of you who helped running the meeting by being chair, presenter and/or participant.

We are looking forward to seeing you all next year!

With kind regards,

The EURL fish and crustacean team