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Muscle spoilage in Nephrops

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Ágrip á íslensku:

Í verkefninu var unnið með humariðnaðinum á Íslandi við að greina orsakir og skilgreina lausnir til að draga úr vöðvadrepi í leturhumri. Slíkt vöðvadrep hafði aukist mjög á síðustu árum án skýrrar ástæðu. Í upphafi var gert ráð fyrir að líkleg ástæða vöðvadrepsins væri *Hematodinium* sýking í stofninum en slík sýking hefur valdið töluverðum áföllum í skoska leturhumarstofninum. Staðfest var að ekki voru tengsl milli *Hematodinium* sýkingar og vöðvadreps. Í framhaldinu varð því að breyta áherslum verkefnisins. Með ítarlegum formfræðirannsóknum á leturhumri tókst að tengja vöðvadrepið við ensímvirkni í hepatopancrea leturhumars. Byggt á þeim niðurstöðum var unnin skilgreining lausna til að draga úr tíðni vöðvadrepsins. Með bættri kælingu og meðhöndlun með ensímhindra hefur tekist að draga verulega úr vöðvadrepi í leturhumri.

Lykilorð á íslensku: *Humar, vöðvadrep, kæling, gæðastýring, ensím*

Summary in English:

This project was carried out in close association with the Icelandic *Nephrops* fishing and processing industry. The aim was to define reasons and propose solutions to reduce the muscle spoilage in *Nephrops*. Such muscle spoilage had increased significantly during the last few years without any know reason. The original hypothesis of the project was that there might be a correlation between infection of the parasite *Hematodininum* and muscle spoilage. Such parasitic infection has resulted in lower quality products in the Scottish *Nephrops* industry for the last decade. In the project it was confirmed that such infection is not the underlying factor for the muscle spoilage. This resulted in change of direction in the project. Based on morphological analysis of *Nephrops* it was observed that the muscle spoilage was correlated with enzyme activity in the hepatopancrea. Based on this observation it was possible to propose a code of practice to reduce the onset of the muscle spoilage. The code of practice is based on improved chilling and use of enzyme inhibitor during the storage of the *Nephrops* from catch to frozen product.

English keywords: *Nephrops, muscle spoilage, chilling, quality control, enzyme*

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INTRODUCTION

Original AIM of the project

Muscle spoilage in *Nephrops* has increased dramatically in Icelandic waters since 2006. It is even in some cases observed in the majority of the catch. Muscle spoilage (MS) reduces both taste and texture of *Nephrops.* If no action is taken, the consequences can be the loss of important markets. Biological effects of MS on the stock are still unknown. The main aim of this project was to define what causes MS in *Nephrops*. To achieve this, a multidiscipline group of scientist and stakeholders was formed. It was proposed that MS resulted either from *Hematodinium* infection or stress. Following definitions of the causes of MS, environmental, catching, and processing variables should be analyzed to define how they affect the frequency of MS. Based on those data, a code of practice to reduce the frequency of MN would be proposed, both regarding catching management and fishing process. Further the following aims were specified:

1. Defining causes of muscle necrosis (IS: skyrhumar) in the Icelandic *Nephrops* stocks
2. Development, validation and implementation of sensitive molecular diagnostic tools for detecting "skyrhumar"
3. Prevalence of "skyrhumar" and association with environmental and post-capture factors
4. Data analysis and comparison to prevalence in UK
5. Proposal of a code of practice for reducing ratio of "skyrhumar" in commercial products
6. Final compilations

The decapod crustacean *Nephrops norvegicus* (Norway lobster) is a burrowing clawed lobster with muscular tail. In Iceland, *Nephrops* are found in ten main (separated) areas along the south coast, varying in size and importance to the fishery (7).

The global annual landings of *Nephrops* are in excess of 70.000 tons of which approximately a half is from UK waters (29). For half a century *Nephrops* has been an important commercial fishery product in Iceland with annual landings of about 1500-2000 tons (1). Although providing only 3% of the global catch, Iceland has a strong market position. This is mainly due to higher percentage of larger *Nephrops* in the Icelandic catch but also to the reputation of high quality products. The export value in 2005 was approximately of 1 million Icelandic kronas (ISK) per ton[1]. All *Nephrops* From Iceland are exported frozen although export of live or fresh lobster to continental Europe results in the first sale price that is often threefold higher. This is especially the case for the largest lobster. It has been reported that up to 14.000 ISK per kilo are paid by continental restaurants for live large *Nephrops* (15).

It is important for the Icelandic lobster industry to be able to provide products of outstanding quality to maintain its strong position at the higher end of the market. The relatively low market volume of the Icelandic *Nephrops* (~ 2%) makes its position at the high end more fragile if the industry offers products of reduced quality, even for a limited time.

Muscle spoilage

Muscle spoilage in *Nephrops* resulting in e.g. bitter taste and negative texture attributes has been occasionally reported during the years. Such muscle spoilage has been referred to as "skyrhumar" in Icelandic. In 2006, a drastically increase of "skyrhumar" in the catch was observed. In some cases the majority of the catch showed characteristics of muscle spoilage. Stakeholders are very concerned about the situation as it greatly threats the position of Icelandic *Nephrops* at the high end market. It can also quickly affect the quality image of the products that has taken decades to build up in a negative manner.

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[1]Homepage of Statics Iceland

No data has systematically been gathered to estimate the frequency and causes of "skyrhumar". Only by defining the underlying factors for "skyrhumar", measurements can be taken to reduce or eliminate its appearance.

It is alarming not knowing what causes the "skyrhumar" in the Icelandic *Nephrops* stock. Epizootic infection with high rate of mortality in the Icelandic scallop stock (*Chlamys islandica*), has e.g. resulted in a collapse in the scallop fishing industry.

The aim of this three year project was therefore to define the causes of "skyrhumar" and to develop a code of practice to reduce the prevalence of skyrhumar in the industry. It should be stated that, although we all agree on the importance of knowing the biological/biophysical reasons for the recent onset of "skyrhumar", there was a strong emphasize of defining methods to "fix" the "skyrhumar" problem for the industry at first.

Already known causes of muscle spoilage in *Nephrops*

At least two different mechanisms have already been described resulting in muscle spoilage of *Nephrops* :

- parasitic dinoflagellates of the genus *Hematodinium* have damaged commercial stocks of *Nephrops* and other important crustacean.
- idiopathic muscle necrosis (IMN) has also been observed in *Nephrops* from the west coast of Scotland. IMN results in characteristic whitening of the tail muscle within hours of capture with a progression towards complete opacity and onsets of bactermia in days.

In this project the initial hypothesis was that either one or both of those mechanisms were most likely the underlying factors of "skyrhumar".

Hematodinium

Hematodinium was originally described in 1930 as an infecting agent of Green Shore and Harbour crabs (5). Since then it has been reported to infect various crustaceans often in epizootics manners with adverse effects on commercial utilization. In snow crabs it causes a condition known as bitter crab disease (BCD) that renders them unmarketable due to ruining of flavor and texture (6, 27). *Hematodinium* infection results in the death of the host due to histological breakdown of body tissues and organs, haemocyte degradation and marked changes in physiology and biochemistry. The consequences are both a loss of valuable products and a quality decline when captured at early stage of infection. So far, *Hematodinium* has been reported to infect *Nephrops* in UK and Swedish water only. In Clyde Sea, epizootics with up to 70% prevalence has been reported (8). In the Irish Sea prevalence of up to 35% has been reported (4). In Skagerrak prevalence has been reported up to 22% (28). In all cases the infection is seasonal, peaking twice a year (25). Interestingly, infected *Nephrops* show reduced swimming performance and endurance. In addition, the animals emerge from their burrows for significantly longer periods than usual, exposing them for capture by trawling (24, 26). Therefore it can be proposed that *Hematodinium* epizootics and increased amount of capture could interrelate. Interestingly, the capture in Icelandic waters in 2006 was well above average.

Idiopathic muscle necrosis (IMN)

Description of IMN is similar in different crustaceans. This pathology is restricted to the abdomen, often with extensive loss of muscle structure. This is followed by an opportunistic bacterial infection that causes further tissue spoilage. In early stages of the condition individual muscle fibers show opacity. In later stages the opacity progresses to muscle fibre bundles and, finally, to the whole abdomen musculature, resulting in death after a few days. The secondary bacteremia that is also often involved, is indicated by a completely white appearance to the abdomen (not simply white streaking), reddening of the pleopods and a distinct off-odor of the muscle. IMN eventually results in the loss of normal functions of the abdomen, preventing normal swimming of the host. In a study of trawled *Nephrops* in the Clyde Sea 8% prevalence of IMN was observed immediately

after the capture. When re-assessed, four hours later the prevalence had increased to 29%, confirming the rapid onset of IMN.

An important factor for IMN induction in crustaceans appears to be stress. Thus IMN occurs in crustaceans following exposure to stressors such as trawl capture (19, 23), temperature and salinity changes (10), handling, air exposure (30), and starvation (12). If it is possible to define the conditions that prevent the onset of IMN, it would have important consequences for both quality of frozen products and export of living/fresh products.

Prevalence of *Hematodinium* and IMN in the Icelandic *Nephrops* stock

No systematic analysis has been carried out to test if "skyrhumar" could result from *Hematodinium* infection and/or IMN. The description of "skyrhumar" muscle spoilage fits, in many ways, to the visual characteristics previously described for identifying both *Hematodinium* infection and IMN. Significant increased frequency of "skyrhumar" in the spring 2006 resembles in many ways the characteristics of epizootic *Hematodinium* infection. The increased catch in trawls in 2006 could also result from reduced mobility of *Nephrops* since both *Hematodinium* and IMN reduce swimming performance of the animal. It is of major importance to define the underlying factors or agents inducing "skyrhumar" in the Icelandic *Nephrops*. When the molecular bases of "skyrhumar" have been defined, measures can be taken to reduce its onset. Further, by understanding the relationship of the prevalence and defined factors of capture (e.g. location, depth, temperature, time of year, and time of trawling) or post-capture handling (e.g. air-, light-, heat-, and chemical exposure), advisory measures for catch management and handling may be defined if significant correlation is obtained.

PROCEEDINGS

Defining causes of muscle spoilage (skyrhumar) in the Icelandic *Nephrops* stocks

In the original application, we proposed two possible mechanisms likely to induce the muscle spoilage, i.e. *Hematodinium* infection and Idiopathic Muscle Necrosis (IMN) due to stress. Both conditions were known to affect the consumption quality of the tail meat. Both conditions have been defined in the Scottish *Nephrops* stocks by Dr. Douglas Neil and coworkers (18, 21).

Standardizing detections methods between Iceland and Scotland

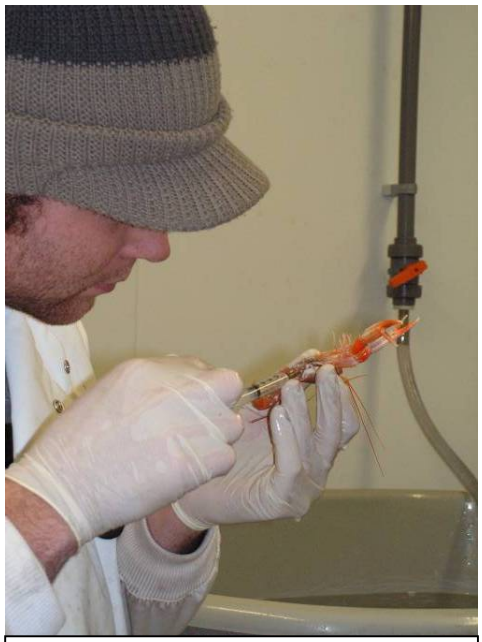
The first part of the project was aimed on defining the causes of muscle necrosis in the Icelandic Nephrops stock. Our study focused on checking for correlation between "skyrhumar" and either *Hematodinium* infection or IMN. To gain full understanding of these two mechanisms of muscle spoilage the project manager visited the University of Glasgow. It resulted in collaboration between Icelandic and Scottish researchers in the field of *Nephrops* biology and genetics. The aim of the visit was to master several techniques developed for analyzing the onset of muscle spoilage due to either *Hematodinium* infection or IMN in the field. These techniques are subjected to objective estimation of the infection. Therefore it was important to learn these techniques from persons already trained to perform it. Furthermore it was necessary to compare the test results of detection performed by the teacher and the student. The following procedures were learned and standardized during the stay at University of Glasgow:

1. **Laboratory work:**
    a. Standard blood sampling procedure
    b. PCR analysis of *Hematodinium*
    c. ELISA analysis of *Hematodinium*
    d. Pleopod indexing
    e. Blood smearing analysis
    f. Fixation of tissue samples
    g. Cell culturing of *Hemtodinium*

2. **Anatomical measurements:**
   a. Carpace length
   b. Sex
   c. Molting stage
   d. Gonad indexing

3. **In field work:**
   a. On board sampling
   b. Color indexing for *Hemadinium* and IMN
   c. Live transportation to aquarium

A detailed review of the currently ongoing *Nephrops* R&D projects were observed in a day long workshop with Dr. Douglas Neil group and with a visit to Millport Marine Station where we met up with Dr. Jim Atkinsson.



Nick Beavers extracting blood from a Nephrops at the Millport Marine Station in Scotland

During the stay notable differences were observed in the texture of the muscle necrosis in fully cooked lobster meat from heavily *Hematodinium* infected lobster and IMN lobster, compared with what we had already observed in the Icelandic "skyrhumar". Moreover it was clearly noted that the aim of the studies in Glasgow were strongly focused on the biological aspects of the infections instead of more practical aspects for the industry. The issue of "skyrhumar" was discussed with represents of the Scottish industry and according to them a large epizoological-like onsets of muscle spoilage like the one observed in Icelandic waters 2006 have not been reported. The scale of the problem in Iceland was such that one might argue that if similar outbreak would happen in e.g. the Scottish water the industry would definitely note it.

Development of standardized semi-quantitative indexing of "skyrhumar" prevalence.

Development of a reliable method for a semi-quantitative indexing of the "skyrhumar" prevalence was of major importance to the project. Further it was of importance to ensure that the method could be applied in difficult settings (e.g. at sea) by non-specialized persons after relatively simple training procedure. In this manner it would be possible to gather data about the prevalence of skyrhumar throughout the Icelandic *Nephrops* industry which is mainly located in Höfn, Vestmannaeyjar and Þorlaksshöfn.

Such method could then be used for further developments e.g. molecular diagnostic tools for the detection or for scanning of morphological changes that could be linked to the onset of "skyrhumar".

The following protocol was developed for indexing:

Part A. Preparation

1. *Nephrops* are randomly collected (no less than 20 individuals).
2. All collected *Nephrops* are tailed.
3. The tails are taken and cut (with scissors) from front end to the back fin resulting in V-shaped tail.
4. Ten tails are put on a plate and cooked at 800W for 4 min in a microwave oven.
5. The tails are allowed to cool for 5 min.

Part B. Indexing

The tail meat is indexed into four groups given a value ranging from zero to three. The following criteria's were used for each group:

0 = the muscle is firm when touched. The texture of the muscle is firm. No mushiness at all is detected at the "skyrhumar" hotspots that are typically the front part of the muscle (lying closes to the carapace and therefore to the hepatopancrea) of the animal and around the intestine track lying after the muscle tail.

1 = some mushiness is observed when touched. Mushiness is found around the "skyrhumar" hotspots, especially in the front of the muscle and close to the intestine track.

2 = mushiness is clearly observed. Mushiness is detected in the overall muscle but the muscle still has some texture.

3 = the muscle is totally mushy. In this case all texture is away and it is easy to spread the muscle out with the finger.

Based on this indexing it is possible to calculate the "skyrhumar" index for the group analyzed. The "skyrhumar" index therefore has a range from 0 to 3 were 0 indicates no "skyrhumar", 3 indicates all "skyrhumar" and 1.5 indicate 50% "skyrhumar".

It also permits calculation of % not showing "skyrhumar".

We have used the "skyrhumar" indexing as a main tool in this project. It is easy to setup and the only tools needed are scissors and microwave oven.

Correlation of prevalence for "skyrhumar" and morphological factors.

A large effort was put on scanning for morphological factors that could be correlated with the presence of "skyrhumar". Of special interest was to find factors that could indicate presence of "skyrhumar" without having to cook the muscle. It was also of importance that such methods could be applied with ease. As the "skyrhumar" indexing is a quite effective way to estimate the prevalence of "skyrhumar" we decided that complex analytical methods such as histopathology of the muscle structure would not be suitable for analyzing the onset. Following aspects were therefore analyzed (**Error! Reference source not found.**):

Table 1. Aspect analyzed in search for correlation of "skyrhumar" and morphological factors

Aspect	What looked for	Difference observed	Conclusion
Shell color	Color difference	No	
Shell pattern	Patterns, spots, texture of shell	No	
Shell thickness	Difference in thickness	Some indication	Soft lobster
Pleopod indexing	Aggregates, cells, parasites	No	
Blood smearing (microscopy)	Aggregates, cells, parasites	No	
Blood color/precipitation	Different clearness, color, precipitation after centrifuge	No	
Organ structure	Difference in organs, e.g. swollen, color change	Some indication	Hepatopancrea burst
Breakage of the lobster	Broken carapace, tail, lack of limps	Some indication	Carapace broken/squeezed
Indication of bacterial infection	E.g. color of pleopods, clearness of blood, smell, curved tail muscle, white spots in muscle	No	
Texture of uncooked meat	Stiffness, puncture, smearing, cutting	No	
Color of tail muscle	Color (clear, opaque), clean	Some indication	Brown liquid on the muscle
Vitals Muscle reflex	Reflex of live lobster	No	

As can been seen in table 1, some indication were found of correlation between morphological factors and "skyrhumar" in four aspects. It should be noted that no aspect showed perfect correlation to skyrhumar. First it seems to be a noticeable higher amount

of “skyrhumar” in soft-shelled *Nephrops*. Secondly, it seems that a noticeable higher amount of “skyrhumar” in *Nephrops* that have its hepatopancrea burst. Later when the color of the tail was analyzed it was found that if brown liquid was on the muscle it often resulted in “skyrhumar” when boiled. Furthermore increased amount of “skyrhumar” was noted in *Nephrops* with broken or squeezed carapace. The four aspects that connected to “skyrhumar” can all be linked together in an interesting manner. Hepatopancrea is much likelier to burst in soft-shelled Nephrops due to mechanic forces during the fishing and processing. Broken carapace is clearly a major factor for bursted hepatopancrea. Further the brown liquid found on the muscle connected to increased amount of “skyrhumar” was traced to bursten hepatopancrea.

Sample collection

During the project samples were collected from commercial vessels from both Höfn and Vestmannaeyjar in Iceland. Samples from all defined areas were collected and the prevalence of “skyrhumar” estimated. Samples were collected both onboard (from live individuals) and from processing plants. Moreover a comprehensive spatial sample collection was carried out in May 2007 during the annual *Nephrops* testing of Hafrannsóknastofnun. 2000 samples were extracted from 10 areas (Figure 1). Tissue sample was taken from each individual and both biometrics and environmental variables logged in standardized manner. DNA was extracted from all samples and arrayed in microplate format. This has laid the foundation for the largest DNA and tissue sample library from *Nephrops* that we know of. The aim of the sampling was to be able to do PCR screening of the *Hemotodininum* parasite.

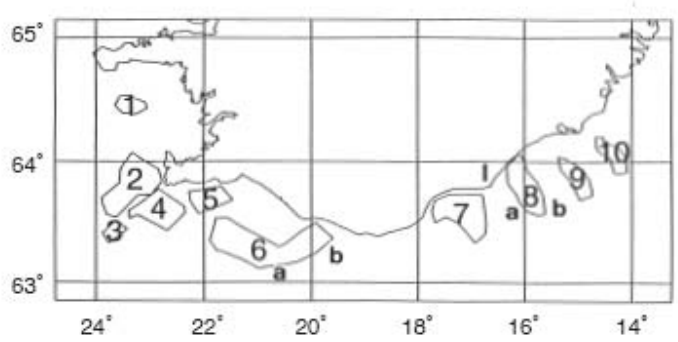


Figure 1. Location of Nephrops fishing areas at Iceland.

Comparison of prevalence for *Hematodinium*, IMN or "skyrhumar".

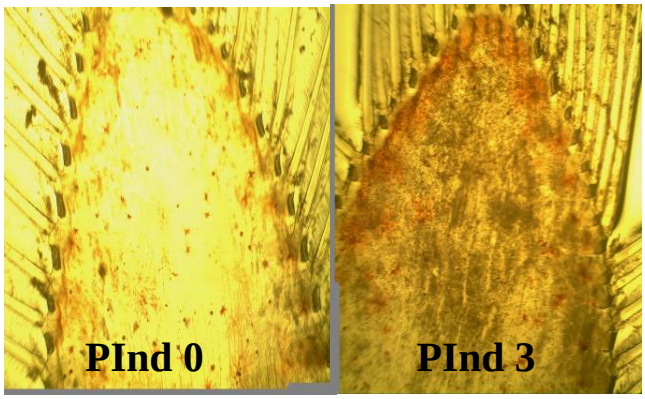


Figure 2. Pleopod indexing. Showing individual without infection (PINd O) and heavily infected individual (PInd 3)

Hundreds of samples were screened for *Hemadodinium* infection using Pleopod indexing (Figure 2). High frequency of infection was observed using the indexing or 71%.

Interestingly *Hematodinium* infections judged by Pleopod indexing increased with the size of the *Nephrops*. The average indexing for *Nephrops* with Carapace length varying from 50 to 60 mm was Pleopod index of 2.78 compared to 1.82 for *Nephrops* with carapace length between 30 to 40 mm. On the other hand frequency of "skyrhumar" showed a slight trend to decrease with increased size of *Nephrops* (Figure 3).

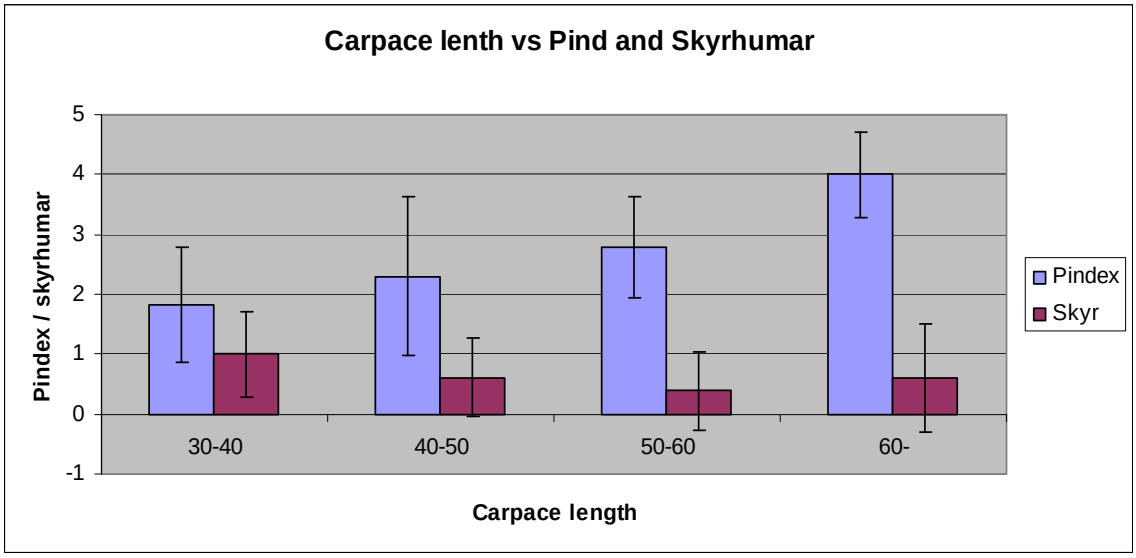


Figure 3. Relationship between size of Nephrops and Pindex and Skyrhumar onset.

No statically significant correlation was found between "skyrhumar" and *Hematodinium* infection based on Pleopod indexing. However this does not exclude the possibility of *Hematodinium* being an underlying factor necessary for onset of "skyrhumar". The pleopod indexing showed a very high frequency of *Hematodinium* infection. However other indicators of heavily infected *Hematotdinium* lobster were not observed. Those include e.g. observation of the parasite in the blood collected from the lobster, cuticle discolouration, and visible aggregates between the membrane and the muscle tissue in the tail muscle. It was therefore decided to do both ELISA screening of blood samples and PCR screening of extracted DNA samples to analyze the prevalence of *Hematodinium* infection and its correlation with "skyrhumar".

To achieve this, a PhD student at the University of Glasgow, Nick Beevers, stayed in Iceland during the summer 2008 to assist in setting up both ELISA and PCR scanning methods at Matis R&D laboratory. Those methods are now available at the laboratory and can be applied when needed. He also collected his own samples at sea to ensure the same protocols were used as in Scotland. After standardizing the methods in the Icelandic laboratory with positive and negative samples from Scottish *Nephrops*, 200 samples collected in September 2008 were analyzed with ELISA and PCR methods. The observed prevalence of *Hematodinium* infection using ELISA was 1% and isolates from these two lobsters were cultured at Glasgow University. Interestingly all DNA samples resulted in absence of *Hematodinium*. However, positive controls (DNA from infected Scottish Nephrops) gave a strong and clean PCR signal. A special emphasis was put on PCR amplifying the samples which resulted in strong and clean positive signal with the ELISA measurement. Amplification from those samples using PCR was not achieved but culturing of the parasite *in-vitro* allowed for PCR amplification and analysis of the ITS1 region of the ribosomal DNA. Analysis showed the genotype to be the same as that infecting Scottish *Nephrops*. Because of the virulence of the Scottish genotype and its high prevalence around the West of Scotland, important questions arise as to how the parasite is maintained at such a low level in Iceland. Also, the use of PCR on haemolymph from crustaceans is questioned as quality DNA was not amplified, despite

an infection of over 100,000 cells per millilitre. These issues require more attention. It is also pertinent to consider *Hematodinium* as a possible threat to the *Nephrops* fisheries in Iceland.

When the ELISA result for the 1% prevalence of *Hematodinium* was compared to the onset of "skyrhumar" in the same individuals, it became clear that none of the two positives showed any sign of "skyrhumar". However, the amount of "skyrhumar" was 21% when measured 36 hours after catch. No correlation was thus found between *Hematodinium* and "skyrhumar".

A change of direction in the project during the second year.

Our analysis strongly indicated lack of correlation between the *Hematodinium* infection and onset of "skyrhumar". This was however the main hypothesis of the project and most of the defined subtasks were designed around that hypothesis. However, as has been explained above, strong indications were found linking an unknown activity of the hepatopancreas to the onset of "skyrhumar". Due to this fact a thorough literature screening was carried out to see if similar observations have already been described. Such hepatopancreas related muscle breakdown was found to be previously described for whole fresh water prawns (*Macrobrachium rosenbergii*). The breakdown was related to proteinase activity, the adhering hepatopancreas during its autolysis (11). Nip et al. reported that the shelf life of iced fresh water prawns was no more than 3-4 days because of enzyme activity (16). Such spoilage was assumed to be caused by the action of endogenous enzyme. Crustaceans posses high concentrations of digestive proteases, mainly serine proteinases. It is also well known that parasites often increase the amount of proteinase activity of hosts (17, 22). Based on this information a new hypothesis was proposed. It assumes that the muscle spoilage in the Icelandic Nephrops stock was due to proteinase activity segregating from the hepatopancrea of the animal. The following facts also favor the idea of hepatopancreas segregated proteinase activity:

- Frequency of "skyrhumar" seems to be lower if the tail is separated immediately after catching. This approach is generally not used anymore as *Nephrops* are generally tailed in the plant up to 48 hour after catching. In addition only small part of the catch is tailed as the main market is for whole-frozen *Nephrops.*

- In 2007 an Icelandic restaurant bought *Nephrops* from a single catch. Part of it was frozen down as whole the rest was tailed. Frequency of "skyrhumar" was much higher in the whole lobster compared to the tailed ones.

Based on these findings it was decided to change the focus of the project. The new hypothesis became that activity form hepatopancrea segregated proteinase might correlate with the onset of "skyrhumar".

Effect of hepatopancrea on the onset of "skyrhumar".

The first step was to test if hepatopancreas would result in "skyrhumar" if it came in direct contact with the tail muscle. Then, interest was in testing if such induction of "skyrhumar" would be due to a biological active ingredient of the hepatopancrea.

To test this, the hepatopancreas from 20 *Nephrops* were pooled and minced into a liquid form. Half of the mix was incubated at 70°C for 5 min followed by cooling in ice. Such preparation should inactivate all enzymatic activity in the hepatopancrea mix. Meanwhile, the lobster tails were cleaved into two parts in such way that they were only

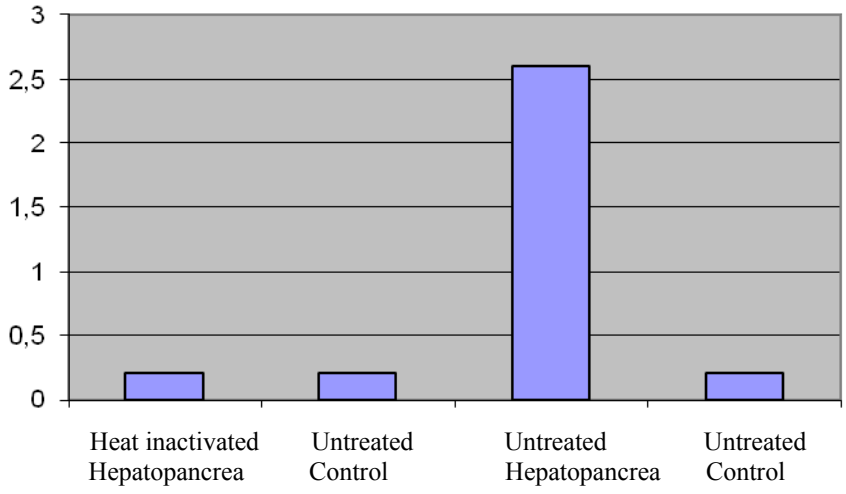


Figure 4. Effect of hepatopancrea mince on onset of "skyrhumar".

joined at the tail. On one part of ten lobster-tails, untreated hepatopancrea mix was added. On the other part of each tail, the heat-inactivated mix was added. The samples were then kept for 24 hours at 4°C. Subsequently the samples were tested for "skyrhumar". This experiment gave very clear results (Figure 4). Very low levels of "skyrhumar" were observed on all lobster-tail parts except the parts were untreated hepatopancrea mix was added. No difference was observed between the untreated controls and the samples with the heat-inactivated hepatopancrea mix. This result clearly indicated that the "skyrhumar" onset can be linked to enzymatic activity in the hepatopancrea.

Defining the activity of the hepatopancrea mix

The next step was to analyse what kind of enzymatic activity was in the hepatopancrea mix. First it was tested if the hepatopancrea mix had collagen activity and if heat-inactivation would eliminate such activity. To do this, 6% collagen in 1.5 ml eppendorf-tube was used. Three drops of either untreated or heat-inactivated hepatopancrea mix were added just prior to solidification of the collagen gel. The mixture was kept at 4°C for 24 hours. In the case of heat-inactivated hepatopancrea mix the collagen mixture became solid indicating a lack of all collagen-degradation activity while the untreated hepatopancrea mix resulted in liquid form of the collagen mixture due to collagen-breakdown. It was therefore concluded that a collagen-degradation enzyme activity in the hepatopancrea mixture exists. Similar results have been observed in freshwater prawn (16). The addition of 10% w/v EDTA in the hepatopancrea mix was as well tested. EDTA is a well-know food grade inhibitor for metal-dependent enzyme activity (3).Addition of EDTA did not hinder the collagen activity measured by collagen-solidification tests. However, the addition of EDTA to the hepatopancrea mix reduced the onset of "skyrhumar" when tested on tail muscle (Figure 5)

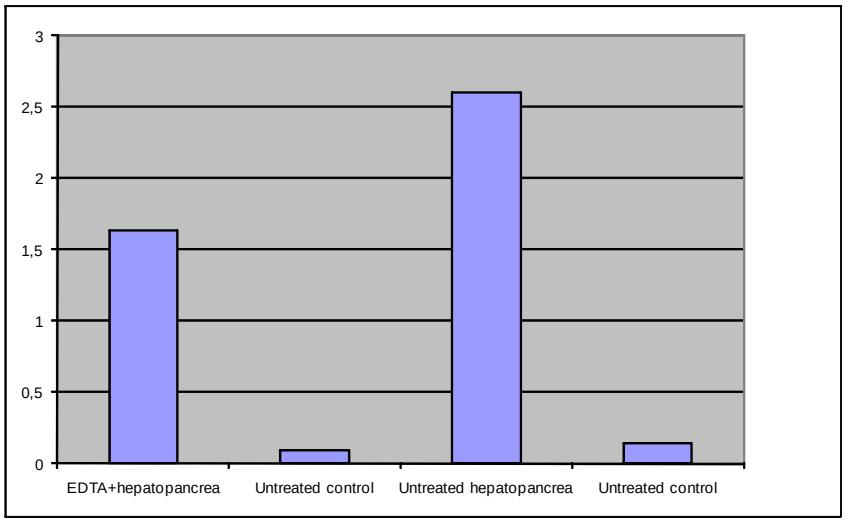


Figure 5. Comparison of EDTA treated and untreated hepatopancrea on the onset of "skyrhumar". Untreated controls refer to one part of the tail that was not smeared with hepatopancrea mince.

Based on these results it was assumed that EDTA treatment of *Nephrops* might reduce the onset of "skyrhumar". However, it was still unclear if whole and fresh *Nephrops* would allow for reasonable uptake of EDTA from a solution. This data indicates that "skyrhumar" results from mixed enzyme activity as EDTA did not seem to block the collagen activity.

Mixed-enzymatic activity of in the hepatopancrea has therefore been identified as the explanation of the onset of "skyrhumar". Similar observations have been published for other crustaceans such as; Crawfish, Kuruma prawn, Rock lobster, Freshwater prawn, Crayfish, and Blue swimmer crab (20). Although considerable effort has been given in understanding the basis for such increased enzymatic activity in the hepatopancrea in different crustaceans, no results have been published which revealed the cause.

In an agreement with all partners of the project it was decided to focus the effort on developing techniques to reduce the hepatopancrea derived enzymatic activity in Icelandic *Nephrops* instead of focusing on defining the molecular aspect of the increased activity. This was mainly due to an urgent need to reduce the onset of "skyrhumar" that was already starting to cause disturbance at both export markets and the Icelandic market.

Effect of harsh treatment of the carapace on the onset of "skyrhumar".

As has already been pointed out, is seems likely that harsh treatment of the carapace may result in increased onset of the "skyrhumar". The idea is based on the fact that a higher "skyrhumar" onset was observed for soft-shelled or broken *Nephrops* when the catch was analysed. To confirm this hypothesis the following experiment was set up. Total of 80 *Nephrops* were randomly picked from the catch when it arrived onboard. 40 of those were taken and their carapace squeezed in such a manner that it did not break. The other 40 were kept aside. Both groups were kept in ice for 8 hour and subsequently analysed for "skyrhumar" index. The result was very clear (Figure 6).

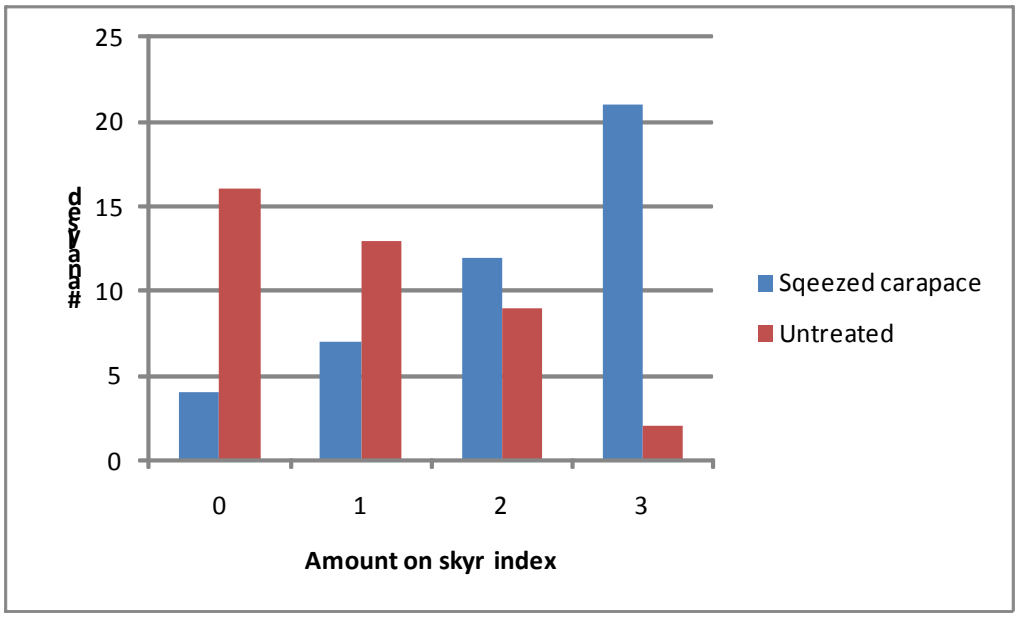


Figure 6. Effect of harsh treatment of the carapace on the onset of "skyrhumar". . Nephrops with squeezed carpace vs. untreated. Number of Nephrops rated 0, 1, 2 or 3 on the skyr index.

The onset of "skyrhumar" was much higher for the *Nephrops* with squeezed carapace. By squeezing the carapace without breaking it, the "skyrhumar" index went from 0.925 to 2.14. It was also striking how many *Nephrops* exhibited total mushiness (3 on the index) after the treatment. This strongly indicates that harsh treatment of the catch can result in

increased onset of "skyrhumar". Based on this it may also be assumed that enzymatic activity in the hepatopancrea is powerful enough to result in "skyrhumar" as long as it is able to leak to the muscle. Similar results were also observed when *Nephrops* were taken from the processing plant and treated in the same manner.

Effect of immediately tailing lobster at sea.

If the hypothesis of segregation of proteinase from hepatopancrea is correct it would be logical to assume that removal of the head (containing the hepatopancrea) from the tail should reduce the onset of "skyrhumar". There are strong indication that this is the case e.g. for lobsters that are tailed onboard immediately after catching (e.g. lobster tails at the black market seems to have lower onset of "skyrhumar"). To test this hypothesis, an experiment was carried out. Fresh lobster tails were collected directly from the boat. Those tails were tailed immediately after the catch arrived onboard, kept at 0°C in chilled seawater for 24 hour and then frozen at -24°C. For comparison, frozen lobster tails from the same catch from the processing plant were collected. Those lobster tails were tailed and frozen approximately at the same time as those which were frozen and tailed at sea. Frozen, whole-lobster from the same catch, were collected from the processing plant. When the "skyrhumar" indexes for all three samples were compared, striking results were observed (Figure 7). The "skyrhumar" index for the *Nephrops* tailed immediately after

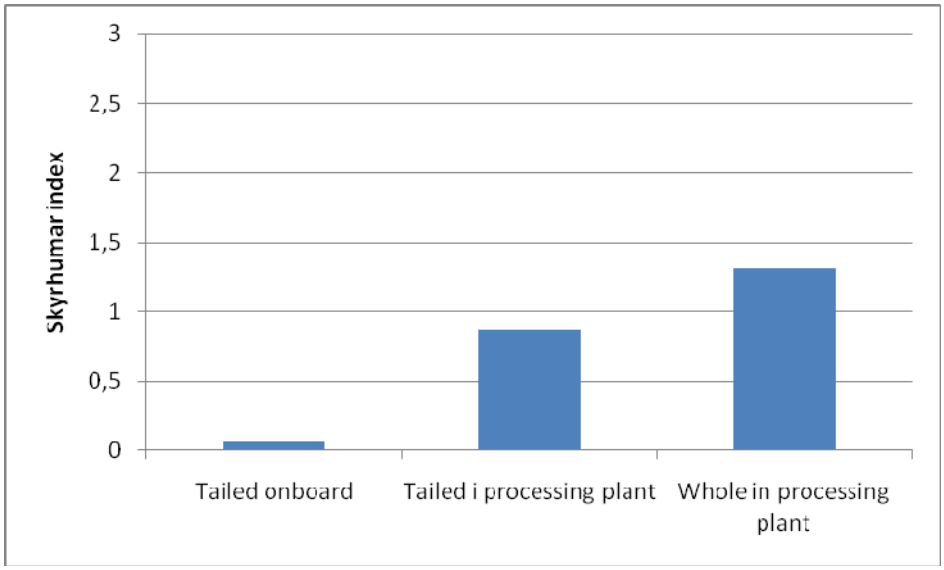


Figure 7. Comparison of "skyrhumar" in lobster tailed onboard or in the factory and of whole-lobster. All samples are from the same catch. 40 individual werea analyzed for each group.

catching at sea, was very low (0,07). *Nephrops* tailed at the processing plant from the same catch revealed 12.25 fold higher onset of "skyrhumar". Finally, the frozen whole-lobsters from the same catch revealed 18.31 fold higher onset of "skyrhumar" compared to those tailed onboard.

Based on these results, the hypothesis was confirmed: Removal of the hepatopancrea from the muscle tissue immediately after catch will considerably reduce the onset of "skyrhumar". The fact that lobsters from the same catch, tailed approximately 48 hours later, reveal more than twelvefold higher onset of "skyrhumar" indicates that the onset might be time dependent.

Time-dependency of "skyrhumar" onset.

The next logical step in the project was therefore to test it the onset of "skyrhumar" was time-dependent. Additionally, the aim was to estimate where, in the process, the onset of "skyrhumar" was being initiated and how it progressed. This included gaining knowledge of whether the onset would accumulate over period of time or if it had a sharp induction phase. Furthermore, the aim was to test if the onset would at some time-point achieve steady state.

The first test focused on how prolonged time in the onboard reception, after harvesting but prior to further processing onboard, affected the onset of "skyrhumar". Typically lobster is exposed to harsh conditions in the reception. Often it contains large amount of catch which might cause increased breakage and squeezing of the *Nephrops*. Further, chilling of the catch is usually not applied in the reception; temperature of the catch in the reception is therefore quite similar to the temperature of the surrounding. The core temperature of the *Nephrops* at harvesting was measured the same as the surface temperature of the seawater (8-10°C). The experiment was executed from a long haul (6 hours) to see if "skyrhumar" would be detected at the time of arrival. Hauls typically do not get longer than 6 hours when fishing for *Nephrops*. This should therefore be the most

extreme conditions for pre-capture onset of "skyrhumar". Interestingly, "skyrhumar" was not detected in the catch at the time on onboard arrival (Figure 8)

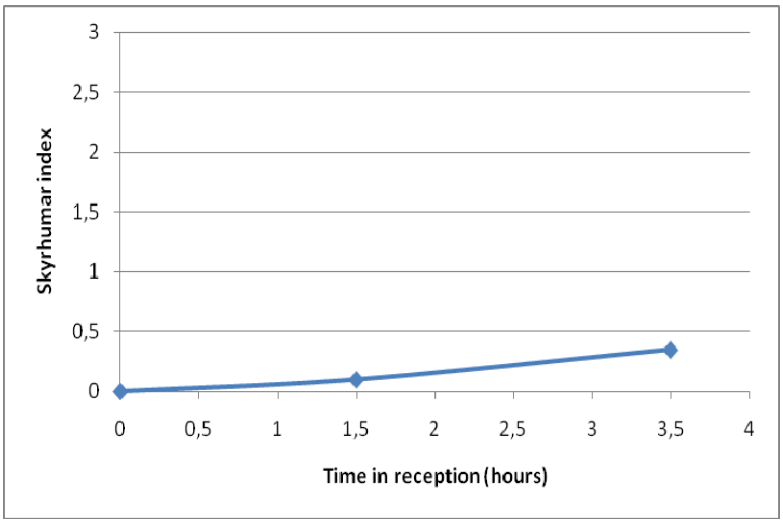


Figure 8. Time dependency of the "skyrhumar" onset during the stay in the onboard reception.

When "skyrhumar" index was measured in *Nephrops,* which stayed for 1.5 hours in the reception, an increase in "skyrhumar" onset was detected. This trend was further confirmed with analysis of *Nephrops* that were kept in the reception for 3.5 hours. It is not uncommon when processing of the catch is onboard, that it takes up to 5 hours to remove all the catch from the reception. This is especially the case when fishery is good and when all the *Nephrops* are tailed onboard.

Next the onset of "skyrhumar" was traced from onboard arrival until fully processed in the processing plant 62 hours later. The onset of "skyrhumar" was as well compared to the temperature in the storage tub. A steep onset of "skyrhumar" was observed during the first 8 hours from catching. This can easily be visualized by plotting the % of *Nephrops* not showing "skyrhumar" as a function of time (Figure 9).

From this plot is also observed that the "skyrhumar" onset seems to approach steady-state after approximately 12 hours from harvesting. Further, increased onset could not been seen at the time point when the cooling capacity of the ice in the tube terminates

(approximately 36 hours). In this graph the number of *Nephrops* that do not show any signs of "skyrhumar" is observed. It does therefore not indicate whether *Nephrops*

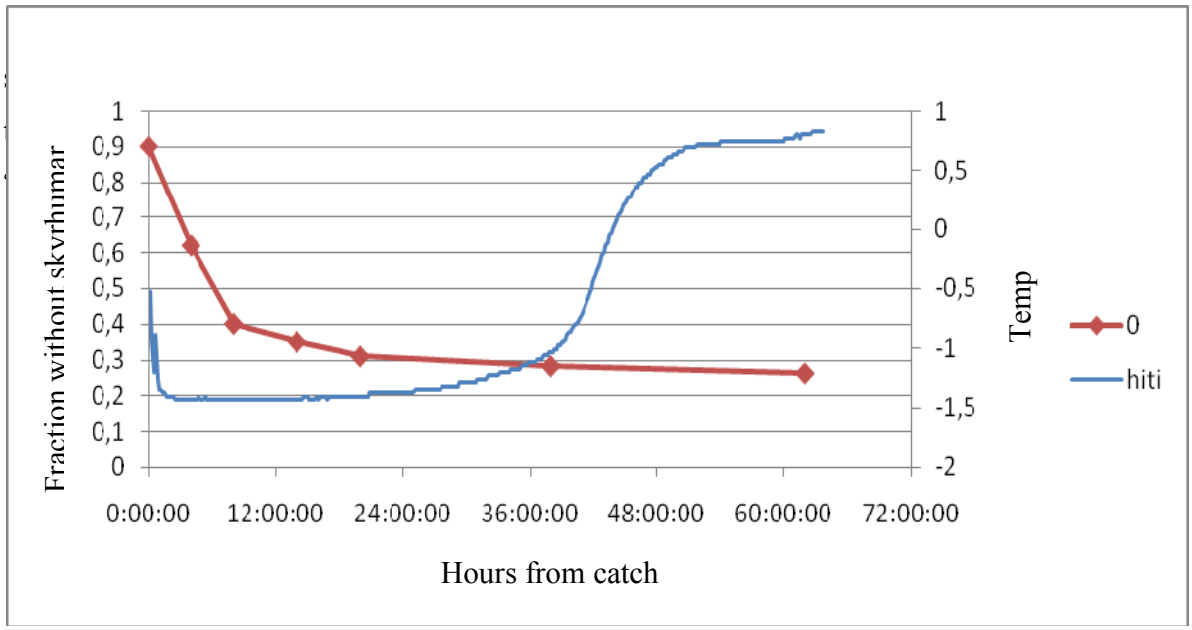


Figure 9. Time dependency of the "skyrhumar" onset from catch to processing. Absence of "skyrhumar" prevalence (%) in *Nephrops* as a function of time (hours) from catch to processing (red line). Storage temperature as a function of time (blue line).

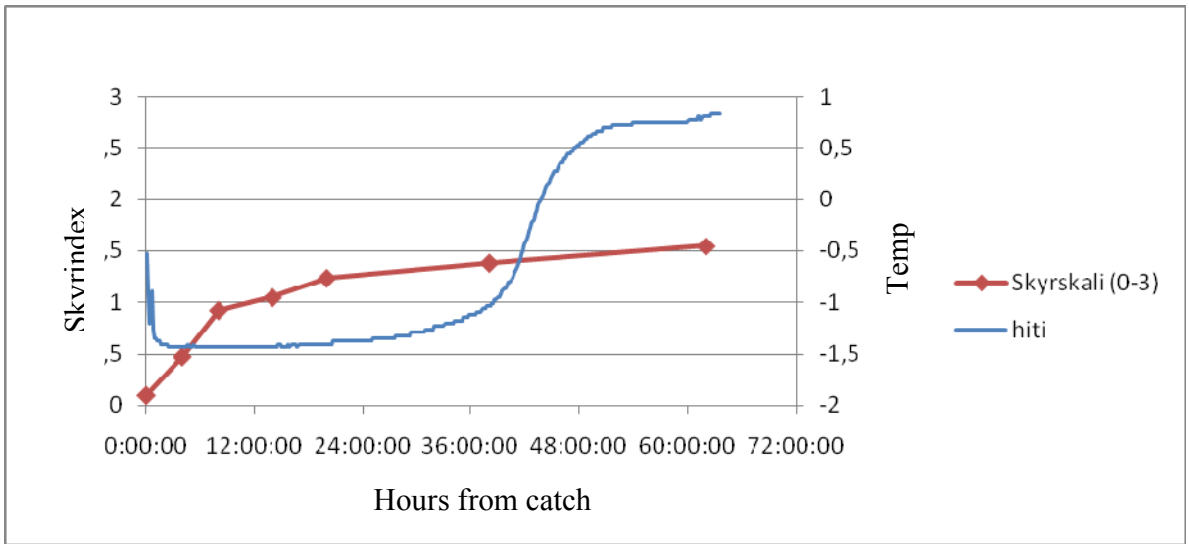


Figure 10. Time dependency of the "skyrhumar" onset from catch to processing. Onset measured as skyrindex.

As can been seen in Figure 10, the "skyrhumar" increases in a steep manner for the first 24 hours but levels off for the rest of the time. When the number of *Nephrops* ranked in each group from 0-3 on the "skyrhumar" index were further analysed, it was confirmed that "skyrhumar" seemed to progress from mild to severe with time (Figure 11).

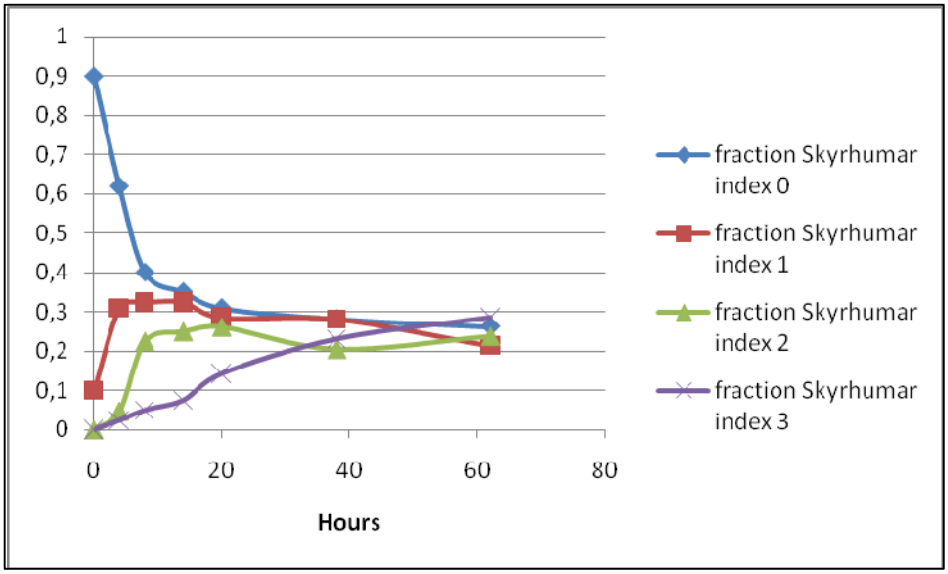


Figure 11. Time dependency of the "skyrhumar" onset from catch to processing. Onset categorised according to rating (0-3) in the "skyrhumar" indexing system.

As the number of *Nephrops* without any sign of "skyrhumar" approaches steady state, it was concluded that the onset of "skyrhumar" was not infectious during storage. Rather, it was assumed that the onset were due to some pre-conditions in given fraction of the *Nephrops*.

The time-dependency of the "skyrhumar" onset was tested several times during this work. Similar trends were always seen. This can e.g. been seen when two fishing trips in July and September 2008 are compared (Figure 12).

These observations indicate that the onset is very steep in the first few hours after the catch with a relatively steady state afterwards. These results indicate that possible solutions are limited to relatively short time span.

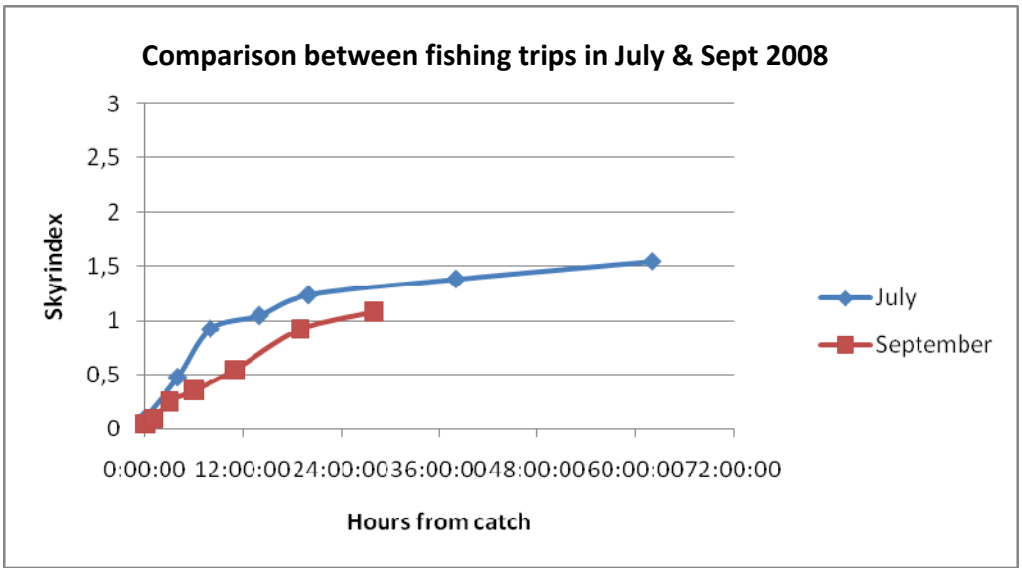


Figure 12. Comparison in "skyrhumar" onset between two fishing trips, in July and in September. "Skyrhumar" onset measured by skyrindex.

Ways to stop/reduce the onset of "skyrhumar"

As the onset of "skyrhumar" is due to enzymatic degradation of tail muscle, there should be several physical/chemical approaches to reduce or stop the onset. Below are summarized several identified approaches (Table 2).

Table 2. Possible approaches to reduce the onset of "skyrhumar".

Approach	Based on
Blanching	Inactivate enzyme with increased temperature
Freezing	Essentially stop enzyme activity with freezing
Chilling	Inducing sub-optimal temperature for enzyme activity
pH	Change pH in environment to value outside optimal range for enzyme activity
Tailing	Remove the source of enzyme by removing hepatopancrea
Protein inhibitors	Adding chemicals to reduce/block enzyme activity
High pressure	Inactivating enzymes by high pressure treatment
Keeping alive	Reducing the enzyme leakage from hepatopancrea to muscle by avoiding post-mortem conditions in tissues

Reducing "skyrhumar" by blanching.

Similar conditions in whole-crawfish industry have been resolved by using blanching at high temperature early in the processing line (13). Such blanching of prawn seems to inactivate the enzyme resulting in increase of mushiness. Possibilities for blanching whole-lobster. Such approach did not work as the lobster meat will cook (whiten) at temperatures as low as 50°C. Such temperature is not high enough to inactivate the enzyme activity.

Reducing "skyrhumar" by chilling/freezing.

Having identified that the "skyrhumar" phenomenon was due to enzymatic breakdown of the muscle tissue, the chilling-pathway onboard came as well into focus. Enzymes typically show highest efficiency at its environmental temperature. In this case it was most likely that the enzymatic activity would be at maximum at about 6-12°C. Based on this, it was likely that the activity could be reduced by decreasing the temperature or even increasing it.

The effect of freezing whole-lobster at -20°C immediately after catching was tested. The "skyrhumar" index of the frozen batch was compared to a batch processed onboard for storage in the regular ice/seawater mixture. Further, the effect of keeping batches at un-chilled and super-chilled (ice/salt/seawater) conditions were compared (Figure 13).

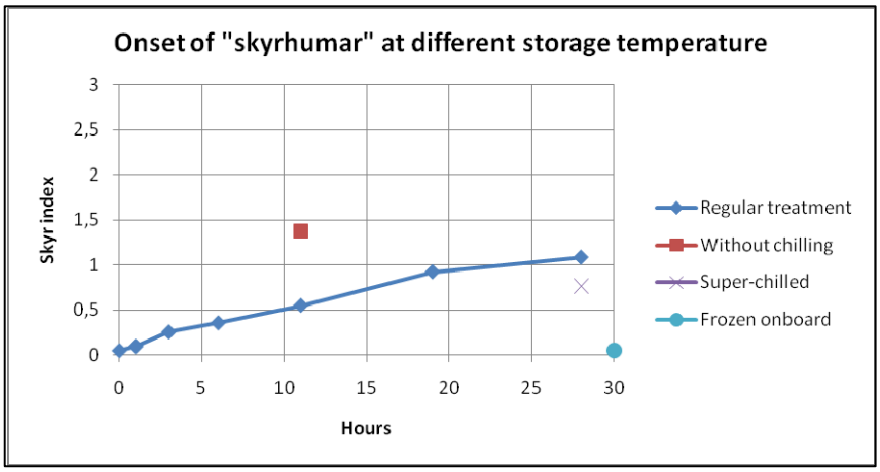


Figure 13. Effect of temperature treatment on the onset of "skyrhumar" measured by skyr index. Change in skyr index with time.

Based on these observations it was confirmed the chilling/freezing was a major factor in reducing the onset of "skyrhumar". When no chilling was used 2.5 fold increase in "skyrindex" was observed when compared to regular chilling. *Nephrops* kept under superchilling conditions had 1.4 fold lower onset of "skyrhumar" compared to the regular chilling. As was expected, the whole-lobster frozen onboard showed very low onset of "skyrhumar" or 20-fold lower than the one kept under regular conditions. These results confirm therefore that freezing the lobster onboard immediately after catching might eliminate the "skyrhumar" problem. However such freezing would have to be achieved in the short time period prior to steep onset of "skyrhumar" in the catch.

The effect of super-chilling was further looked into by using a small-scale brine freezer. The brine freezer can effectively chill down to −21°C using fully saturated sodium-brine. The brine freezer has a digital temperature control allowing for accurate controlling. The effect of keeping lobster in super chilled brine at -8°C was tested. At -8°C the lobster was in a semi-frozen state in the brine. The reason for not using lower temperature was due to that the lobster will become very fragile during further handling when it is brine-frozen. Samples were analyzed after 12 hours at either -8°C superchilling, normal chilling (~-1.5°C) and without chilling (~11°C) (Figure 14).

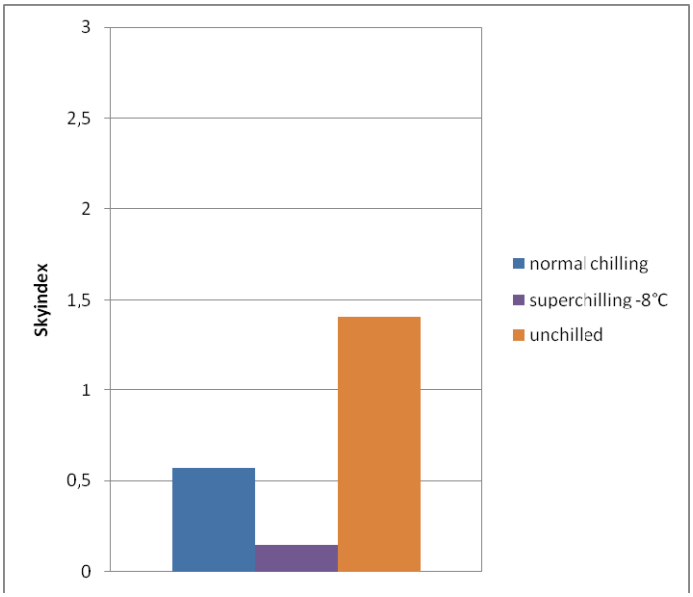


Figure 14. Comparison between normal chilling (-1.5°C), superchilling (-8°C) and no-chilling (~12°C) of whole *Nephrops* on their skyr-index.

Lobster kept at -8°C showed 4-fold reduction in “skyrhumar” compared to lobster kept in regular conditions and a 10-fold lower “skyrhumar” index when compared to lobster that was left unchilled for 12 hours.

The possibility of cooling the catch down to -8°C by dipping in superchilled brine for 30 minutes was further tested. The catch was then either iced in layers without addition of seawater or kept in ice/seawater slush (Figure 15).

Same amount of “skyrhumar” was observed when the superchilled products were subsequently added to the normal ice/seawater slush chilling. If superchilled products were iced in layers without addition of seawater to the tubes the “skyrhumar” was reduced approximately 3-fold. This may be explained by more effective heat-transfer in the ice-water slush setup resulting in the superchilled products to approach the temperature of the slush in quick manner.

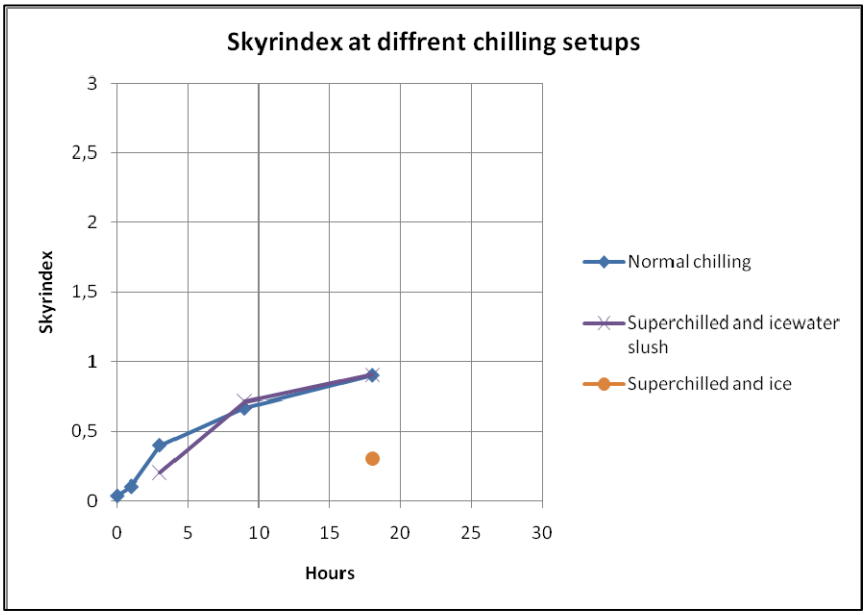


Figure 15. Effect of superchilling in brine freezer at -8°C and subsequently keeping in either ice/seawater slush or using ice.

The analysis of the influence of chilling on the onset of "skyrhumar"data clearly confirms the importance of respecting a proper cool-chain from the arrival of the catch onboard until frozen for market.

Reducing "skyrhumar" by tailing.

As has already been shown, it is possible to reduce the onset of "skyrhumar" simply by removing the carapace from the tail immediately after catch. It is however clear, based on the data above confirming the time dependency of the onset, that such tailing would have to be carried in the first 4 hours or so after catching.

Reducing "skyrhumar" by keeping lobster alive

Enzymatic activity similar to the one described in the previous analysis is often connected to post-mortem stage (14). If this is the case, it can be argued that keeping lobster alive after catch should postpone the onset of "skyrhumar". To test this lobster was kept alive for 12 hours onboard after catching using continuous flow of fresh seawater. The lobster was then tail and compared to lobster from the same catch that was kept under standard condition. 4.7 fold reduction of "skyrhumar" was observed in the batch which was kept longer alive.

Reducing "skyrhumar" by changing pH of the solution.

One way to reduce activity of proteolytic enzymes is to shift the environmental pH out site its optimal range. Based on data describing similar findings in different crustaceans it was decided that the such approach would not work as the activity has generally broad range of activity or between pH 5.5 to 10 (9). It would not be possible to keep the catch at such pH without affecting its general quality.

Reducing "skyrhumar" using hydrostatic high pressure inactivation

Hydrostatic pressure inactivation of enzymes in fish is well known methodology (2). It is likely that such treatment could greatly affect activity of enzymes in the flesh. However,

it was decided that such methodology is currently not applicable onboard the fishing vessel. This possibility was thus not looked further into.

Reducing "skyrhumar" using protein inhibitors.

Proteinase activity was observed as the main agent for the "skyrhumar", there might be a possibility to inhibited the activity using protein inhibitors. Various kinds of protein inhibitors are available. Well known proteinases are iodoacetic acid (cysteine protease inhibitor), PMSF (inhibits serine and some cysteine proteases), pepstatin A (inhibitor of aspartyl proteases), leupeptin (inhibitor of cysteine and serine proteases) and EDTA (metalloprotease inhibitor). All except EDTA have in common to be both expensive and non-food grade chemicals. EDTA was therefore chosen for trials (in concentration likely to be within the EU regulations for allowed levels in food).

For the purpose to find out if this was feasible, the chemical products already available for the industry were scanned. Such products are usually aimed on reducing melanosis progression and microbial spoilage. One chemical was found that contained EDTA. The trade name is Hasenosa. The product contains; Sodium Metabisulfite (E223), Citric Acid (E330), Ascorboric Acid (E300), EDTA Acid (E385) (< 1%), Sodium Bicarbonate (E500ii), Glycerol (E-422), Maize starch and anti-caking agent (E-504-i). Based on communications with the producer they included EDTA primarily to reduce the microbial spoilage.

Hasenosa was chosen for treatment of "skyrhumar". The reason was twofold, firstly, the chemical product was already accepted by EU directories and secondly, it contained bisulfite which was currently used onboard. As a first test, it was decided on adding 1 kg of Hasenosa into each tub (containing approximately 150 kg of lobster) (The given protocol assumes dipping 100 kg crustaceans in 3 kg of Hasenosa). This was done as our aim was to incubate the lobster in the Hasenosa mixture instead of dipping. The onset of "skyrhumar" was then checked after both 24 and 48 hours in the tubs (Figure 16).

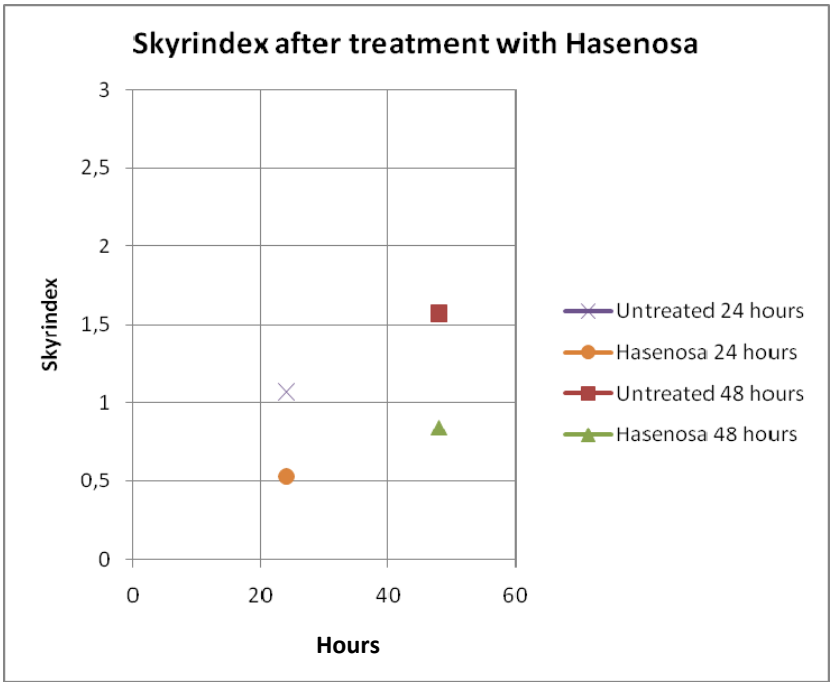


Figure 16. Effect of Hasenosa incubation on skyrindex. Change in skyrindex with time (hours).

Interestingly a two-fold reduction in "skyrhumar" was observed with the combination of Hasenosa/Ice water slush compared to the regular Metabisulfite -ice-water slush setup. A noticeable trend of less severe onset of "skyrhumar" was also noted. This was indicated

with reduced number of individuals showing "skyrhumar" at either stage 2 or 3 (Figure 17).

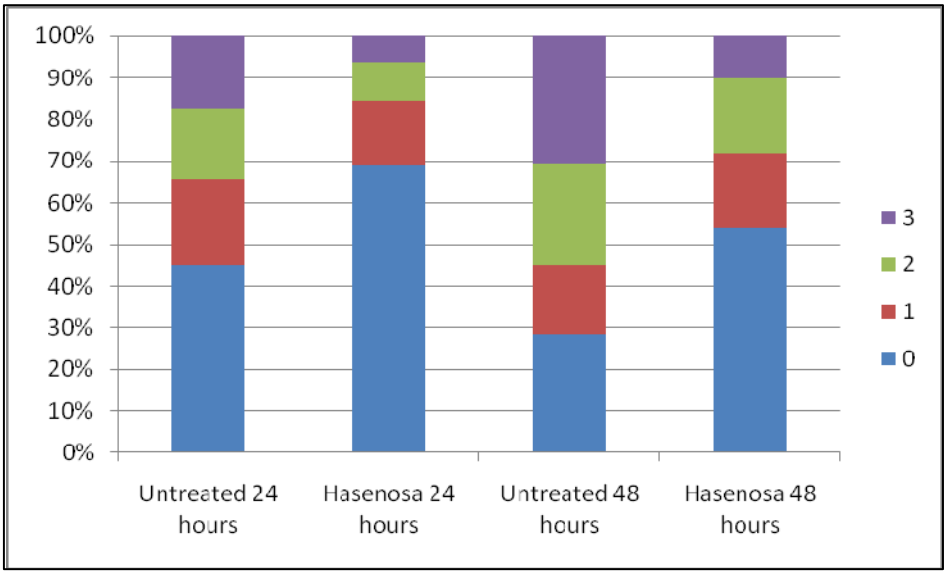


Figure 17. Effect of Hasenosa incubation on "skyrhumar index". Onset categorised according to rating (0-3) in the "skyrhumar" indexing system.

As skyrhumar in stage 1 will not greatly affect the consumer quality of the products while "skyrhumar" in stage 2 and 3 will do so, it is possible to represent the effect of the Hasenosa treatment in form of improved consumer quality (Figure 19):
Hasenosa incubation of the catch is therefore shown to increase the overall quality of the lobster. Based on the experiment the treatment with Hasenosa increased the fraction of lobster being of high consumption quality (0+1) from 48% to 72% when the samples were analyzed after 48 hours of storage in tubs.

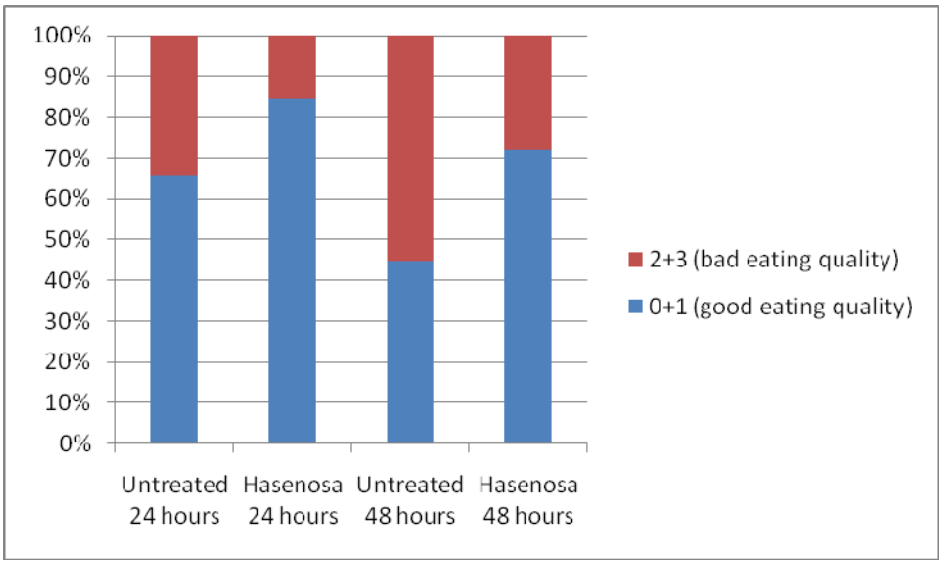


Figure 19. Effect of Hasenosa incubation on "skyrhumar index". Represented as consumer quality indexing.

As this experiment gave a promising result for the industry it was decided on testing it in real life circumstances, that is, let the fishermen themselves treat the lobster onboard with Hasenosa. To do this, 1 kg packages of Hasenosa was prepared. Such package contained the right amount of the chemical to treat the amount of lobster typically put into each storing tub. After adding ice/water slush to the tub the Hasenosa bag was opened in the water. Using this method it was both possible to reduce variations of the amount used and also this approach ensured that the fishermen never come to a direct contact with the chemical. Lobster treated by the fishermen with Hasenosa was analyzed in a blind test setup by two different evaluators in separated tests. The tests were carried out after arrival to the fishprocessing facilities 48 hours after the catch (Figure 20).

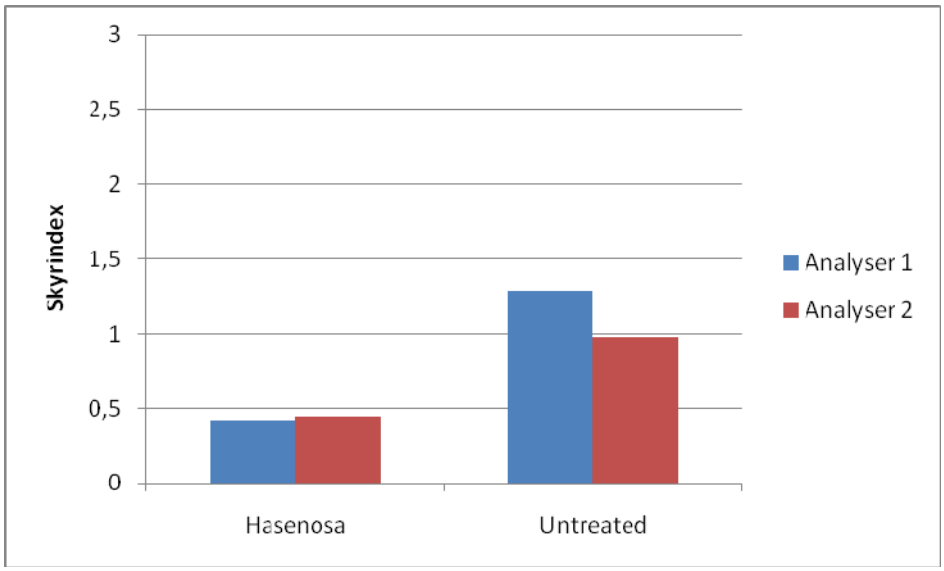


Figure 20. Effect of Hasenosa when used by regular fisherman. "Skyrhumar" indexing was executed blindly by two separated analysers.

In both cases the Hasenosa treated sample showed much lower onset of "skyrhumar" compared to the one treated in regular manner with "metabisulphite"

It should be noted that all experiments with Hasenosa treatment were done on a catch fished at the "Eldeyjar" area and the "Jökuldýpi" area. These are the two most western areas of the Icelandic *Nephrops* fishing grounds (see Figure 1). To ensure that Hasenosa would allow for similar effects in other areas, catch from "Breiðamerkurdýpi", one of the biggest lobster fishing ground at the east area, was treated with Hasenosa. Again, fishermen were asked to replace typical metabisulfite treatment with Hasenosa, keeping everything else
the same in the processing. The onset of "skyrhumar" was tested when the catch arrived at the processing plant 24 hours after harvest (Figure 21).

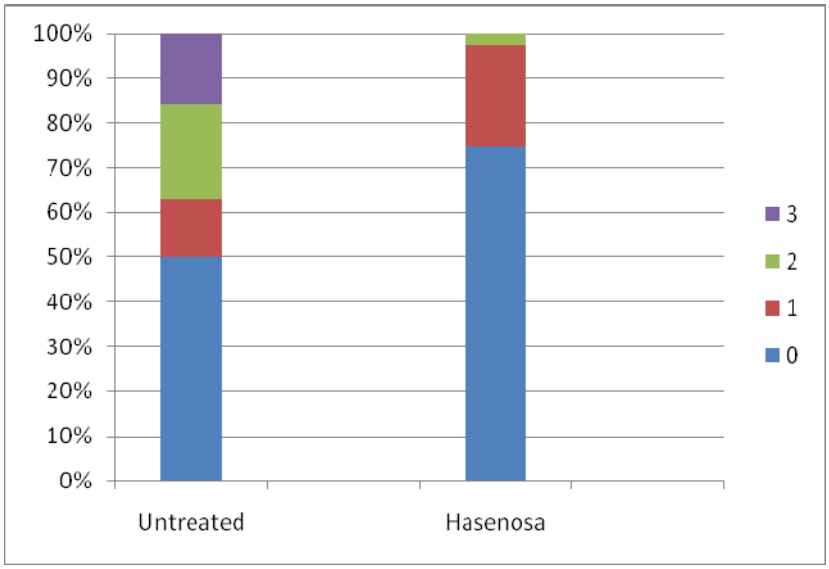


Figure 21. Influence of Hasenosa treatment on "skyrhumar" in catch from the east area measured by skyrindex (0-3). Distribution of catch treated with Hasenosa vs. untreated in skyrindex categories.

Similar reduction of skyrhumar was observed as previously reported for the two western areas. This confirms that Hasenosa treatment can assist in reducing the onset of skyrhumar in the Icelandic *Nephrops* fishery.

Temporal and spatial analysis of the "skyrhumar" onset.

One of the main aims of the research was to see if correlation could be observed between the onset of "skyrhumar" and either spatial or temporal variables. To do this the onset of "skyrhumar" was extensively measured when the catch arrived in the processing facilities. Strong correlation between "skyrhumar" and time of the year have not been observed. However there is an indication that the onset might be a bit lower during May-June than in other months (Figure 22). It is however not easy to do this type of analysis as it is not possible to ensure that each ship is always at the same area at each time-point. For the last couple of years there has been a strong trend in migrating the vessels from the east areas (May-June-July) to the west area (Aug-Sept-Oct). Statistically significant difference has though not been found between areas. It should be pointed out that "skyrhumar" analysis in the processing facilities have not been carried out at a fixed time-point from the harvest. As the onset show great time-dependency this might result in absence of correlation due to large deviations in the analysis.

These analysis however confirm that onset of "skyrhumar is relatively stable between the years 2007-2009 and it is observed in all fishing areas.

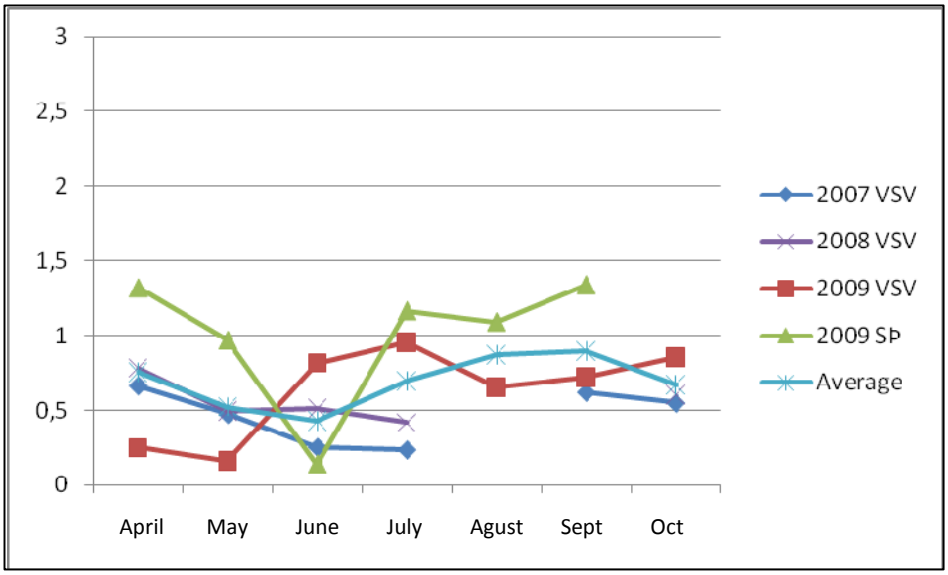


Figure 22. Temporal onset of "skyrhumar" in Icelandic waters during the period of April-October in 2007-2009. Prevelance of "skyrhumar" measured by skyrindex. VSV=Vinnsustodin, SÞ=Skinney Þinganes.

CONCLUSIONS

The major goal of the project, to reduce the onset of “skyrhumar” in the Icelandic fishery, was achieved. The project had a major change of direction when it was confirmed that the original hypothesis of “skyrhumar” being correlated with either *Hematodinium* infection or idiopathic muscle necrosis was not true. However a low frequency of infection was observed for *Hematodinium*. It is therefore of importance to monitor the progress in Icelandic waters as it might be of importance if the frequency rises to the level observed in Scotland. All the participants in the project agreed therefore that it would be necessary to change the focus of the research project. It was observed after morphological analyses of the *Nephrops* containing “skyrhumar” that the onset correlated with some morphological features. These findings supported a new proposal of proteinase activity contributing by the post-mortem segregation of enzymes from the hepatopancreas. A thorough review of the literature revealed that similar observations have been reported for various crustaceans. It was confirmed that "skyrhumar" results from enzymatic degradation of the muscle. It was as well confirmed that such activity was heat-labile and was partially due to collagenase digestion. Time dependency for the "skyrhumar" onset was confirmed. Steep onset during the first few hours from catch was observed, after that it levels off. The amount of “skyrhumar” in each individual increased during storage while frequency of new onsets levels of. This suggests that the onset is dependent on pre-existing factors. It was also confirmed that harsh physical conditions strongly increase the risk of "skyrhumar" onset. Major effort was on reducing the enzyme activity. More effective cooling of the catch than generally was used decreased the onset. This may be explained in terms of shifting the temperature to a range that is not optimal for the enzyme activity. Freezing the whole-lobster on board resulted in major decrease in "skyrhumar". This is however not applicable as the time period prior to the steep onset “skyrhumar” is short. Tailing the lobster onboard seems an effective way to reduce the onset but such tailing has to be carried out in the period prior to the onset. As the main markets of the Icelandic *Nephrops* are for the whole-lobster, it was of importance to define practical approach for reducing the onset of "skyrhumar". The onset was reduced

notably by combination of effective ice/seawater sludge cooling and the use of the food grade protein inhibitor EDTA. A code of practice has been introduced to the industry. Currently it is used by all major companies in the industry, resulting in significant reduction of "skyrhumar" in the Icelandic catch.

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