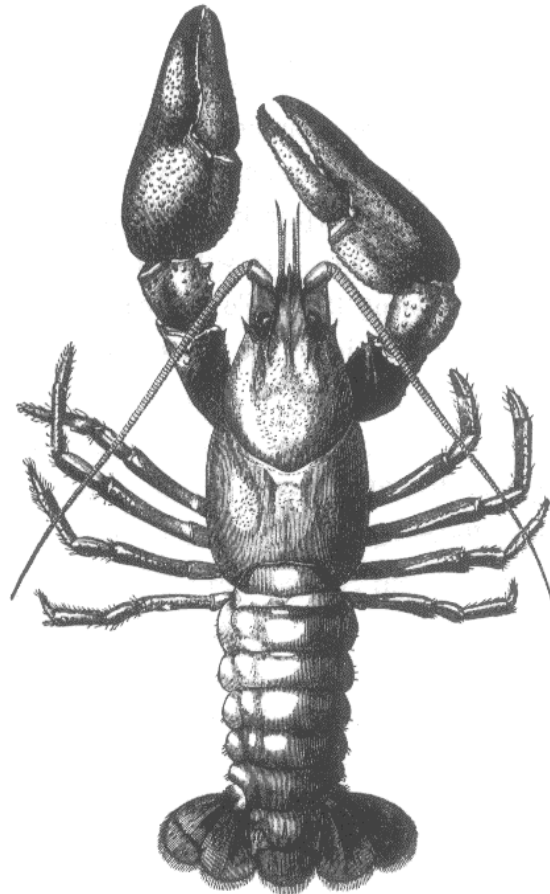


**Growth and macronutritional requirements of signal crayfish,
Pacifastacus leniusculus (Dana) in aquaculture**



Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität
zu Kiel

vorgelegt von
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Kiel
2004

Referent/in: Prof. Dr. Harald Rosenthal

Korreferent/in: Prof. Dr. Hansen

Tag der mündlichen Prüfung: 13. Juli 2004

Zum Druck genehmigt: Kiel, den 15. Juli 2004

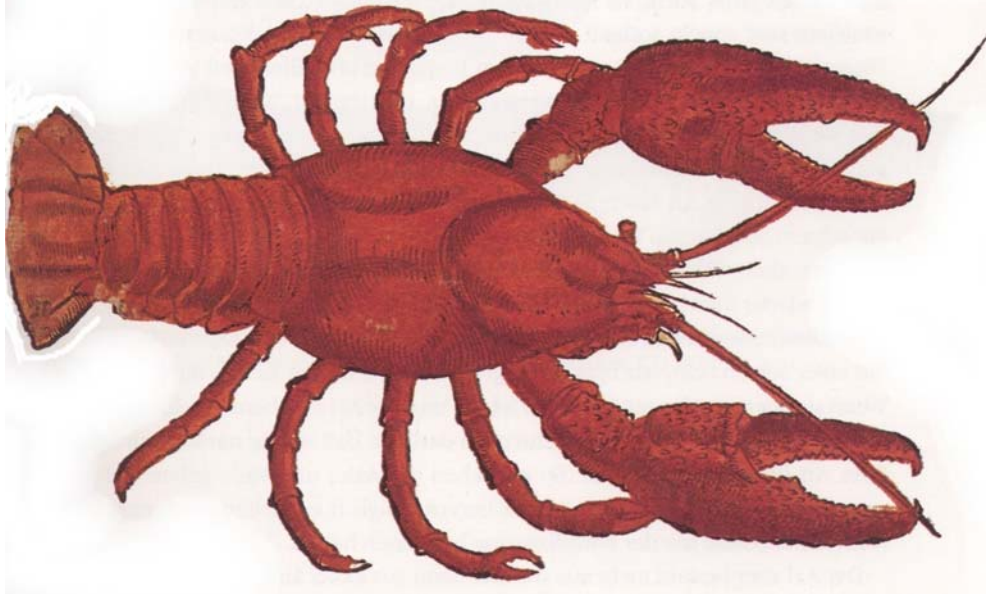
Krabsen.

CXCIII

Von dem Fluß oder Süßwasserkrabs.

Astacus fluuiatilis. Flußkrabs/Gemeiner Süßwasserkrabs.

Von seiner gestalt/vnd mancherley geschlächte der thieren.



Der Cancer ist mehr kalt als warm und hat mehr Wärme von der Erde als von der Luft. Er liebt den Tag und die Nacht, weil er vorwärts geht wie die Sonne und rückwärts wie der Mond. Er hat gesundes Fleisch, und der Gesunde kann ihn ebenso essen wie der Kranke, außer wessen Magen kalt und bestoppet ist. Wer Verdauungsschwierigkeiten hat, für den ist der Krebs eine zu kräftige Speise, weil er ihn schwer verdauen kann. Deshalb ist er für ihn als Speise ungeeignet. In seinem Kopfe aber findet sich ein Fett von frischer Kraft, das crebezus smer genannt wird. Nimm davon und füge eine gehörige Menge Butter dazu und knyt dies zusammen. Wer im Gesichte und um die Nasenlöcher quedelechte hat, so als wollten Schmerz und Geschwüre dort Pusteln bilden, der soll sich in der Nacht an den betreffenden Stellen oft damit einreiben. Wenn er dann morgens vom Bett aufsteht, soll er sich diese Salbe mit Wein aus seinem Gesichte waschen, und er wird eine schöne Gesichtshaut bekommen, so daß sich dort auch keine Pusteln mehr bilden.

**Ja, mach nur einen Plan
Sei nur ein großes Licht!
Und mach dann noch 'nen zweiten Plan
Geh' n tun sie beide nicht.**

Berthold Brecht, Ballade von der Unzulänglichkeit

**Yes, just you make a plan;
Just be a shining light!
And then make still a second plan
But neither one will work**

Berthold Brecht, The song about inadequacy

Acknowledgements

This study was carried out at the Fisheries Research and Aquaculture Station, Finnish Games and Fisheries Research Institute (FGFRI), in Evo, Finland from January 2001 to May 2004.

I want to express my deepest appreciation to my supervisor research professor Kari Ruohonen, PhD, FGFRI, Turku for giving advice and guidance whenever needed and for having the patience to explain the statistics to me. I also have to thank him and the staff of the Evo Fisheries Research and Aquaculture Station for providing very pleasant working conditions and a really nice atmosphere in Evo.

My special thanks to Anja Inkiläinen, M.Sc. Jouni Tulonen, M.Sc. Tero Ahvenharju, Chris Karppinen, M.Sc. Esa Erkamo, Raimo Vilkmán, Pekka Jokela and all the other colleagues of the Evo Fisheries Research and Aquaculture Station. Without them this study would not have been possible. Thanks also go to the laboratory staff of Lammi Biological Station for supporting me and allowing me to use their lab. My warmest thanks go to all the unmentioned colleagues and researchers who have supported me with their work, their questions and their enthusiasm.

Sami “Vesku” Vesala has become a close friend. His patience and support concerning various computer problems always was a big help and he provided the mental distraction I needed when working in Evo.

Special thanks to my supervisor Prof. Harald Rosenthal, Institute of Marine Sciences, Department of fisheries biology, Christian-Albrechts-University Kiel, for enabling me to carry out this study and supporting my work.

My warmest thanks also to Paula Henttonen, PhD, Department of applied zoology, University of Kuopio, for bringing me into contact with the Evo crayfish research group and introducing me to the subject of crayfish study.

Very special thanks go to Ari “Lennu” Mannonen and Japo Jussila, Crayfish Innovation Centre. Their support as well as their entertainment was often essential and always welcome. Both of them have had their share of pain with me and I have to thank Lennu especially for some essential support during his time in Evo.

All those supporters and helpers whom I unintentionally forgot to mention here get my warmest appreciation.

I am very grateful to my parents Angela and Joachim Wolf who never stopped supporting me and always helped me to get along. Not only for financing parts of my studies I owe them my warmest thanks. They provided me with the courage to face the challenges that were connected with me looking for my own way, they were there, whenever help was needed and their feedback was enormously important. Their encouragement, understanding, patience and support made this work possible.

Finally, I want to thank my wife Miia for her love and understanding. Her support in recent years was crucial for the success of this work. Her endless patience was important to me as well as her feedback and her will to cope with my psychotic behaviour.

My wonderful children Joonatan and Sofia have deserved a very special mentioning. Their smile and love were essential for my well-being and I hope I will never have to neglect them again as much as in the last year.

In 2001, German Academic Exchange Service (DAAD) granted the financial support for this study.

A scholarship granted by the German National Academic Foundation (Studienstiftung des deutschen Volkes) financed this project in the years 2002 and 2003.

Further support was granted by Finnish Games and Fisheries Research Institute and Lammi Biological Station.

Tampere, 20.05.2004

Für Miia, Joonatan und Sofia

Schön, dass es euch gibt!

Abbreviations

c l	Carapace-length
DM	Dry matter
dw	Dry weight
FM	Fishmeal
FO	Fish oil
N	Number of replicates
OVAT	One variable at time
ps	Pseudocomponent
SE	Standard error
SD	Standard deviation
ST	Starch
WS	Wheat-starch
ww	Wet weight
y.o.	Years old

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2 Introduction

2.1 History of freshwater crayfish in Central and Northern Europe and recent developments

In many parts of Europe crayfish have played a very important role as a part of freshwater fauna and for commercial exploitation. In the late 19th century crayfish caught in Sweden, Finland, Russia and other eastern European countries were exported to central Europe in large numbers. The markets demanded higher and higher numbers of crayfish (Dröscher, 1906, Floericke, 1915). High catches of crayfish in Central and Northern Europe faced a sudden drop in the late 19th century (*Picture 1*) as a result of the crayfish plague caused by the Oomycete fungus *Aphanomyces astaci*. Due to transports of living crayfish and missing hygienic regulations the new disease could spread rapidly from southern Europe, where it had occurred first, to central and northern Europe. The consequences were devastating. Catches declined rapidly and natural populations vanished within a few years without ever having a chance to regenerate. European astacids do not show any resistance to this disease. Other reasons that have caused declines of the native European crayfish populations are dam construction, draining and pollution (Hager, 1996).



Picture 1: “The Great” (*der Große*) fleeing the city of Nörenberg (Insko, Poland) in Pomerania. Crayfish from local lake Enzigsee were among the most important economic factors in the area. The Poem describes the loss of this profitable business as consequence of “the Great” leaving town (occurrence of crayfish plague?). Notgeld (emergency paper money), City of Nörenberg, Germany, 1921

At the same time, the role of crayfish in their environment became more and more understood and the complexity of their anatomy and morphology was described for the first time in a masterpiece of scientific work by Thomas Henry Huxley. His book “The crayfish. An introduction to the study of zoology”, published in 1879 was a milestone for the understanding of crayfish anatomy and morphology as well as an important description of crayfish behaviour and physiology. Thanks to Huxley, most of the old knowledge about crayfish was preserved and survived many years in which crayfish as a native inhabitant of European water bodies was almost forgotten.

Restoration of the declined crayfish populations by stocking lakes and rivers with native *Astacus* species from healthy populations failed due to reappearance of the plague (Lowery and Holdich, 1988, Ackefors, 1999). The signal crayfish *Pacifastacus leniusculus* (Dana), which is native to northwestern North America, was introduced into European lakes and rivers in the 1960s to replace the populations of noble crayfish *Astacus astacus* (Linné). Before this, several unsuccessful trials with other species like *Orconectes limosus* were carried out (Lowery & Holdich, 1988).

Looking very similar to the commonly known European noble crayfish *Astacus astacus* and inhabiting the same ecological niches, the signal crayfish *P. leniusculus* was soon seen as a suitable candidate to restore crayfish populations in European water bodies. The introduction of signal crayfish into European waters has been followed by a rapid distribution of the new species and has often led to the extinction of the weakened populations of native crayfish species. Signal crayfish appear to be better competitors than the European species.

In addition *P. leniusculus* shows better tolerance for high temperatures and water pollution and it has higher reproduction rates than European crayfish (Lowery & Holdich, 1988). Nowadays signal crayfish are also known to play an important role as a transport vector for *Aphanomyces astaci* (Hager, 1996; Blanke, 1998).

Within Scandinavia and Finland, where crayfish always had played a very important role in commercial and recreational fisheries, *P. leniusculus* was successfully introduced in the 1960s (Simontorp hatchery, Sweden). Recently these introductions to several lakes and rivers in southern Finland have been based on scientific strategies. Long term observations and mark-recapture experiments have given researchers and communities a better estimation of the status of wild crayfish populations (Jussila, 1993; Mannonen and Henttonen, 1995). Stockings are restricted to areas where no noble crayfish populations can be established and the protection of

existing populations is prioritised. The amount of signal crayfish produced in culture has increased rapidly in Finland throughout the last years both for stocking purposes and for ongrowing to market-size (Mannonen, 1999).

Potential markets for crayfish can be found both in Finland and the rest of Europe (Sweden, France, Germany) (Huner *et al.* 1987, Huner 1989, Nyström and Rönn 1990, Holdich 1993). In Europe, the demand for market size crayfish has been higher than the actual European production of crayfish in recent years. Imports from Asia and the United States have reached 4000 metric tons and are still increasing (Ackefors and Lindqvist 1994).

In Finland and other Scandinavian countries rearing and marketing of juvenile stocklings have gained economical importance in addition to the marketing of adult animals (Westman, 1973). Prices for crayfish have traditionally been high both in Central Europe (Jussila 1993, 1995) and Scandinavia (Holdich 1993, Mannonen 1999), which has encouraged farmers to start crayfish culture.

To encourage research and development of crayfish culture methods suitable for Scandinavian conditions, several independent regional Crayfisheries Management Programs were carried out in Southern Finland's provinces in the late 1980's (Jussila *et al.* 1990, Jussila and Mannonen 1995). The aim was to enable farmers to intensify their aquaculture by usage of indoor farming systems for freshwater crayfish.

However, farming methods suitable for Scandinavian conditions remain poorly developed and so far there has been no commercially viable systems for indoor culture of market size crayfish. Crayfish culture in Finland has therefore been focusing mainly on production of juveniles for stocking purposes (Ackefors and Lindqvist 1994). Methods to produce juveniles have been steadily intensified and as a result crayfish eggs are incubated in a sophisticated, intensive and computerized system (Järvenpää and Ilmarinen 1995, Järvenpää *et al.* 1996). Juvenile culture, 0+ year old (y.o.) stocklings, is intensive with stocking densities being around 100 juveniles per m².

In Central Europe crayfish culture still plays a minor role and the increasing demands of the markets are met by importing crayfish from other European countries and the United States. However, the high value of marketable freshwater crayfish might lead to an intensification of culture attempts in Germany, Austria and other European countries in near future (Westman *et al.*, 1990; Bohl, 1999, Hager, 1996).

In recent years more research has been carried out in Europe to improve the understanding of nutritional needs and rearing conditions suitable for freshwater crayfish (Klostermann and Goldmann, 1984; Ackefors *et al.* 1994; Savolainen *et al.*, 2003; Wolf, 2000; Keller, 1995; Keller and Keller; 1995; Brown *et al.*, 1995; González *et al.*, 1993).

Due to the lack of suitable diets as well as several other factors like hierarchy, stress resistance and interaction influencing the success of any aquacultural intensification, freshwater crayfish culture at its present stage is a combination of extensive and semi-intensive techniques (Lee and Wickins, 1992; Ackefors and Lindqvist, 1994; Mills *et al.*, 1994). The level of intensity is determined by species' characteristics in different parts of its life cycle. The technically most advanced culture methods, developed in Finland (Järvenpää *et al.*, 1996), resemble those used in the finfish culture (Ackefors *et al.*, 1994). In these, eggs are hatched in intensive systems, juveniles are raised in intensive or semi-intensive systems (D'Abramo *et al.*, 1985) and grow-out systems for market-size animals are semi-intensive (Lee and Wickins, 1992; Huner, 1994).

Experimental systems can help to evaluate the commercial and biological potential of a crayfish culture system by assessing a range of physiological responses of crayfish under practical rearing conditions and by gaining more knowledge about suitable aquaculture systems for the animals.

2.2 Moulting and growth

For aquaculture, growth is economically the most important physiological response of crayfish to their environmental conditions. As members of the group of *Arthropoda*, crayfish follow a diversified basic arthropod body plan that consists of a segmented body covered with a jointed protective exoskeleton. In common with all other arthropods, crayfish can increase in length only by periodically shedding the exoskeleton during the process of moulting or ecdysis (Penzlin, 1991). The life cycle of crayfish progresses through a series of moults interspersed by intermoult. For crayfish, as for other crustaceans, growth is a complicated mixture of protein synthesis and cellular proliferation during intermoult and a rapid increase in length and weight at moult (Aiken and Waddy, 1992). The weight gain at moult is normally between 30% and 60% of premoult weight. During intermoult the crayfish substitutes its water contents with tissue growth, and the weight gain during the long intermoult is normally less than 5% of immediate postmoult weight.

Studies on crustacean growth have shown that acclimation, dissolved oxygen concentration, food supply, isolation, temperature, photoperiod, sexual maturity and age could have an effect on weight increment at moult or length of the intermoult (Vonk, 1960; Chittleborough, 1975; Aiken, 1980; Hartnoll, 1983; Botsford, 1985; Speck and Urich, 1969; Tacon, 1996). Stocking density or tank/pond size have also shown to affect growth (Aiken, 1980; Morrissy, 1992; Morrissy, *et al.* 1995; Brown *et al.*, 1995).

In northern Europe, hatching takes place from March to June depending on geographical area and during the first summer the animals undergo several (up to 11) moultings; the actual number is governed by temperature and light intensity (Lowery, 1988).

The weight increment of noble crayfish (*A. astacus*) at moult can be between 33% and 65% under laboratory conditions (Ackefors *et al.*, 1995). Intermoult period has been reported to be 58 days for males and 93 days for females of approximately 9cm total length (TL) noble crayfish (Henttonen *et al.* 1993) or from 40 to 44 days for juvenile noble crayfish weighing less than 4g (Ackefors *et al.* 1995). For signal crayfish in laboratory experiments, Westman *et al.* (1993) obtained a mean length increase at moult of between 4.6mm and 5.5mm for summerlings and 4.5mm to 4.8mm for crayfish in their second year. Small differences in growth of females and males could be detected by Westman *et al.* (1993) as well. They obtained the following length increments at moult in noble crayfish: 5.3mm (0+ y.o.) and 4.7mm (1+ y.o.) for males and 4.0mm (0+ y.o.) and 3.0mm (1+ y.o.) for females. Intermoult period in juvenile marron (*C. tenuimanus*) has been shown to be between 15 and 45 days (Morrissy, 1984), while the weight gain at moult was up to 52% of the premoult weight for animals weighing from 0.04g to 120g.

The type and amount of diet affect the production of the crayfish (Lowery, 1988; Gydemo, 1989; McClain, 1995; Barki *et al.*, 1996) by offering a variety of nutrients required in the growth process and altering the level of cannibalism. The nutrient profile affected both weight gain at moult and the length of the intermoult period in a study carried out by Jones *et al.* (1995) on yabbies (*Cherax destructor*). Furthermore, feeding ratio affected both intermoult period length and weight gain at moult, though the influence on intermoult period was greater in the prawn (*P. elegans*) (Salama and Hartnoll, 1992). Barki *et al.* (1996) showed that feed ratio and distribution affected growth, survival and competition in laboratory reared juvenile red claw (*C. quadricarinatus*).

As the crayfish get older the number of moults per growth season decreases. The timing of the moult cycle is under the control of the crustacean endocrine system and involves the interaction of the X and Y organs and secretion of the hormone crustecdysone. However, the nutritional status of the animal and the conditions of temperature and light under which it is kept, influence the process (Aiken, 1969; Lowery, 1988).

Due to moulting the determination of age and growth of crayfish is difficult since the animals do not retain any permanent features such as the growth rings on the scales of fish.

Therefore, a number of different criteria have been used to describe the size and rate of growth of crayfish. The most common are listed below:

- (1) Carapace length: length of the carapace is measured from the tip of the rostrum to the posterior margin at the dorsal midline. Carapace length plotted against the age is frequently used as a means of expressing the pattern of growth of individual animals over a series of moults.
- (2) Annual instantaneous growth rate (g), which is calculated as a logarithm of weight at the beginning (W_0) and the end (W_t) of a twelve-month period.
- (3) Moults increment: increase in carapace length at moult measured for individual animals.

(Brewis & Bowler, 1982)

The growth of juvenile crayfish does not show any sexual dimorphism that therefore becomes apparent only at sexual maturity in the form of the abdomen and the chelipeds (Hogger, 1984 and 1988; Lowery, 1988).

Crayfish species encounter a very wide range of environmental factors from northern Scandinavia to southern Australia (Lee and Wickins, 1992). Perhaps the most important environmental factors are temperature and nutrition. It has been shown in many studies that the growth rate of any crayfish species is affected by temperature and food sources and this probably explains variations in growth rate of a particular species at different locations (Flint, 1975; Mason, 1974 and 1979; Tcherkashina, 1977). However, precise data on growth rate are difficult to obtain from the field because it is almost impossible to determine the age of an animal picked up at random. This is especially true in the case of juvenile crayfish (Wolf, 2000).

In order to assess the effects of these factors on wild populations it is therefore necessary to combine both field data and experimental laboratory studies.

Comparisons of *P. leniusculus* in Lake Tahoe (Flint, 1975) with a Canadian stream dwelling population (Mason, 1974) show the difficulty of comparing wild populations living in habitats with different temperature profiles. Clearly, there are other factors affecting growth rate because the two groups of animals grew at approximately the same rate, as measured by carapace length of year classes, despite the higher temperature of the stream. Here it becomes obvious that nutrition and other factors have to be taken into consideration in any comparative study.

One of the factors that have been reported to have an effect on growth and survival is stocking density. There are many reports stating that crowding affects the growth and reproduction of a wide variety of both terrestrial and aquatic animals. However, the effect of high stocking densities may simply act indirectly by affecting some other environmental parameters such as oxygen or food availability.

With increasing contacts between the animals at higher stocking densities aggressive interactions become more and more frequent. This is likely to have an influence on crayfish survival because the animals undergo periods of moulting when they are vulnerable e.g. to cannibalism. Furthermore, the animals inhabit holes or refuges and therefore the carrying capacity of any particular water area depends very much on the nature of its ability to provide such shelters (Savolainen *et al.*, 2003 and Savolainen *et al.*, 2004). This is important for the design of ponds and tanks for culture.

In moulting the crayfish lose up to 90 percent of the calcareous material of their exoskeleton and they have to replace it from food and the water they live in. It has been suggested that the lower limit of tolerance of crayfish for calcium is 5mg/l and several workers have proposed that crayfish occur only in hard water draining from rocks, that yield soluble components, such as chalk and limestone (Greenaway, 1985).

Studies with *Orconectes virilis* and other crayfish species have shown that they have a limited tolerance for acid pH, especially during the moult, and that calcium uptake is impaired below pH 5.8 and inhibited below pH 4.0 (Greenaway, 1985).

Aiken (1969) was one of the first authors that reported the major importance of photoperiod on the moult cycle of *O. virilis* when kept under constant temperature and environmental

conditions. Other reports by Armitage *et al.* (1973) and Rice and Armitage (1974) underline the effect of photoperiod on the moult cycle and the seasonal distribution of moults. Recent studies in Sweden (Nyström, 1994) have also shown that there is an influence of light intensity on the survival of juvenile signal crayfish. He found a positive relation between juvenile crayfish survival and light intensity. This was apparently caused by lowered activity of the animals exposed to higher light intensities of 600 lux. However, when comparing growth rates of juvenile signal crayfish *Pacifastacus leniusculus* fed at different times in a 14L:10D photoperiod (Wolf, 2000), no differences could be detected and the animals were observed to adapt to the feeding regime.

The life histories of the species in the family *Astacidae* are adapted to the cold-water habitats they live in. Typically, moulting occurs only during the warmer summer months when feeding can take place, although feeding has been observed for *Austropotamobius pallipes* and *P. leniusculus* even at temperatures less than 10°C (Hogger, 1988).

2.3 Feeding biology and dietary requirements of freshwater crayfish

In their natural habitats crayfish occupy low trophic levels and they feed on aquatic vegetation, benthic invertebrates and associated detritus as well as zoo- and phytoplankton (Ackefors and Lindqvist, 1994; Hager, 1996). These omnivorous feeding habits are one of the key factors for the successful establishment of crayfish culture systems.

Crayfish have been described as “uniquely polytrophic” animals and may be regarded as herbivores, predators and detritivores. Their mode of feeding displays preferences for certain types of diets but also shows acceptance of various diets in accordance with age, season of the year and physiological status (Goddard, 1988). Evidence gained from field and laboratory studies shows that juvenile crayfish feed mostly on aquatic invertebrate prey and adult crayfish feed more on vegetation and detritus.

Early aquaculture trials with different crayfish species relied on the natural biomass production in the culture ponds and the pond vegetation was directly provided for crayfish stocks

(Goddard, 1988). Farmers of American red swamp crayfish *Procambarus clarkii* plant rice, rye grass or millet in order to increase productivity (J. Huner, *pers. com.*).

Nevertheless, lack of detailed information about the macronutritional requirements of freshwater crayfish still limits the development of semi-intensive or intensive cultures of cold-water crayfish species. Recent developments in the culture of other crustaceans, especially penaeid shrimps, lobsters and freshwater prawns, are linked to increased research and understanding of their feeding biology and dietary requirements.

The attempts to intensify the cultivation of crayfish like *Astacus sp.* and *Pacifastacus sp.* in ponds and tanks with insufficient sources of natural food have increased the demand for artificial diets suitable for the needs of freshwater crayfish. Although many diets are already now available for farmed crustaceans most of them are developed and composed for prawn and shrimp. Ackefors *et al.* (1992) were among the first authors working on nutritional needs of *A. astacus* with standard experimental diets.

The production of juvenile crayfish in hatcheries and the subsequent rearing in ponds require supplementary feeding with either cultivated organisms such as small zooplankton, filamentous algae and aquatic plants, or formulated diets that suit the needs of newly-hatched or juvenile crayfish. In the early trials starter-feeds for fish were mixed with alginate to prevent disintegration and fouling. However, artificial diets formulated for fish or shrimps seem not to be really suitable for crayfish as they do not provide all necessary nutrients in sufficient quantities, such as cholesterol and carotenoids, and poor growth as well as insufficient pigmentation has been widely observed as a result of this (Henttonen *et al.*, 1993; Jussila, 1997; Wolf, 2000).

Diets formulated on the basis of current knowledge of crayfish nutrition are still under development but have given promising results in laboratory conditions. They form a basis for further development of a convenient diet for cultured crayfish.

The use of formulated diets has reached the state of successfully maintaining crayfish like *P. leniusculus* under laboratory and culture conditions but the knowledge of dietary and macronutritional requirements as well as of feeding habits and behaviour is still to be regarded as poor (Baum, *et al.*, 1991; Evans and Jussila, 1997; Glencross *et al.*, 1999).

2.4 Objectives and experimental set-up

It was the aim of this study to gain more information about the macronutritional requirements of freshwater crayfish. Facilitating good growth and intensification of aquaculture are important steps towards establishing crayfish culture in northern European countries. For the development of suitable diets for culture purposes it is necessary to gain more information about the dietary requirements of signal and noble crayfish and their energy requirements. The present work focused on three major objectives:

1. To achieve more information of the macronutritional requirements of freshwater crayfish. For this purpose, a number of experiments were carried out focusing on different aspects of diet development.
2. To understand the effects of dietary composition on the animals' body composition. These were studied using measurements of body protein and lipid in response to different treatments.
3. To introduce new methodology to measure short-term of metabolic changes of crayfish in response to different dietary and environmental factors.

The following subchapters give a more detailed description of the objectives of the present work.

2.4.1 Macronutritional aspects

Macronutrients constitute the bulk of the diet and supply energy as well as essential nutrients needed for growth, maintenance, reproduction, and activity. Carbohydrates, fats (including essential fatty acids), proteins, and water are macronutrients (CDC, 2004.) Carbohydrates are converted to glucose and other monosaccharides; fats to fatty acids and glycerol; and proteins, to peptides and amino acids. In the body, glucose can be converted to lipids via lipogenesis; amino acids are either channelled to protein synthesis to form new proteins or catabolised for energy and residual carbon chains may be converted to glucose or lipid. These macronutrients are interchangeable as sources of energy; fats yield 39kJ/g; proteins 24 kJ/g and carbohydrates yield 17kJ/g.

Carbohydrates and fat spare protein. Unless sufficient non-protein resources are available from either dietary sources or tissue stores (particularly of fat), protein cannot be used efficiently for tissue maintenance, replacement, or growth, and considerably more dietary protein is required for positive nitrogen balance in terms of biomass gain.

The objective of a proper diet is to achieve and maintain a desirable body composition and a high potential for physical activity. The daily dietary requirements for essential nutrients, including energy sources, depend on age, sex, height, weight, and metabolic and physical activity.

For good health, body composition must be kept within reasonable limits. This requires balancing energy intake with energy expenditure. If energy intake exceeds expenditure or expenditure decreases, body weight increases. Conversely, if energy intake is less than expenditure, weight is lost.

The development of a suitable diet for crayfish in aquaculture depends on a wider knowledge of the macronutritional composition of desired diets. To achieve this aim, several different tasks were included in this study. A first experiment dealt with the macronutrient composition of suitable diets for crayfish. By carrying out a response-surface experiment the influence of the main raw ingredients fishmeal (protein), starch (carbohydrates) and fish oil (fat) was examined. Based on the findings of the first experiment, further trials dealt with the influence of different binding agents on the diets' stability and availability to the animals as well as the influence of different binding agents on the diets' availability and their influences on growth.

Further questions arising from these measurements were the optimal kind of diet concerning the shape and form and comparison to traditionally given food (fish) to set a baseline for growth. It had to be ensured that the animals did not obtain any other food than the formulated diet offered to gain reliable results about the different diets tested.

This aim was reached by using formulated diet manufactured by myself using known ingredients and binding agents to stabilize the pellets. The diet was pelletised and stable in water for approximately 45 to 60 minutes. A strict regime of the feeding was reached by delivering the food only within a short period. In addition, the experimental tanks were cleaned frequently to remove any disintegrated pellets from the system.

2.4.2 Body composition responses to dietary changes

Crayfish can only grow by moulting. Therefore, changes in body composition as well as their general nutritional status cannot easily be determined by weight and body size.

To ensure possible effects of different diets were not missed in this study, measurements were also carried out to determine the contents of water, protein and lipids in the animals. The amount of water in the animals can be seen as an indicator of the nutritional status or their phase in the moulting cycle. The more tissue is build up during the period of intermoult the lower the contents of water in the animal. Therefore, dry weight of the animals was determined by freeze-drying at the end of experiments. The wet weight and the dry weight were then used to determine the contents of body dry matter and water.

Using samples of the freeze-dried animals, the body protein and lipid contents were determined in additional measurements. Both protein and lipids play a key role in crayfish nutrition and protein concentration is an important indicator of nutritional status. Different diet compositions might lead to different concentrations of protein and lipids of animal tissue (Schrif *et al.*, 1987). Protein concentrations of the animals was measured using a modified micro-Kjeldahl method while the concentration of lipids was determined by using the sulphophospho-vanillin method described by Zöllner and Kirsch (1962). Body composition measurements were carried out in the laboratory facilities of the Evo Fisheries Research Station for the following experiments:

- Effect of different diet compositions on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus* (Chapter 4)
- Effects of different types of diet on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus* (Chapter 5)

The objective of these measurements was to assess the effects of different dietary compositions on the body composition of juvenile signal crayfish to gain deeper information for diet development.

2.4.3 Usage of RNA/DNA ratio as a measurement of growth

Another important aim of my work was the introduction of a new method to measure growth and metabolism of cold-water crayfish. The weight and length measurement alone are insufficient because they always leave room for speculation about water intake and actual tissue-growth during intermoult or the partial or complete loss of legs and chelipeds. Because of these complications in measuring the actual response of crustaceans to different nutritional treatments suitable additional methods were sought to close the gap left by the traditional measuring methods. Measurement of RNA/DNA ratio is one such method and it has been a standard procedure for growth measurement in fish larvae and juvenile fish and the method has widely been used with other crustaceans as well (Clemmesen, 1993; Gorokhova and Kyle, 2002; Vrede *et al.*, 2002). The RNA/DNA ratio comparison would allow direct measurement of physiological responses to environmental influences.

Use of modern technologies and methods like RNA/DNA ratio measurement will enable researchers to gain better information about the status of individual animals as well as the nutritional developments in wild populations. Tests carried out with juvenile animals will certainly be applicable to adult crayfish as well, and development of a non-lethal sampling method for measurement of growth capacity could easily follow the introduction of the RNA/DNA method. The method has been successfully used for fish and some crustaceans but not for crayfish (Edsman, *pers. com.*)

The oxygen consuming break-down of most nutrients produces energy and the basic molecules needed for the synthesis of proteins and nucleic acids (catabolism). These molecules are processed by the animal to increase its biomass in an energy consuming part of metabolism (anabolism).

The objective in aquaculture is to maximise retained energy that maximises growth. Metabolic activities are therefore expected to be high for well growing animals and affected by the availability of different macronutrients. Animals can only grow if the total turnover of nutrients leaves sufficient energy that can be used for anabolic activities like protein synthesis.

Protein synthesis is a process that can be made visible by measuring the amounts of RNA in samples of the organism. While the amount of DNA in cells stays remains fairly constant, the amount of RNA in those cells is highly dependent on the nutritional status and protein synthesis activity (*Fig. 1*). Mitochondrial RNA (m-RNA), transfer RNA (t-RNA) and ribosomal RNA (r-

RNA) can be found in different amounts depending on the level of protein synthesis reflecting the nutritional status. By measuring both the amount of DNA and RNA in the samples and calculating the ratio of RNA to DNA, the nutritional status of the animal can be identified in comparison to others with different nutritional supply. A higher RNA/DNA ratio always stands for a better nutritional status, as it represents significantly higher rates of protein synthesis.

Figure 1 explains the principles of protein synthesis and key role of DNA and RNA in the process.

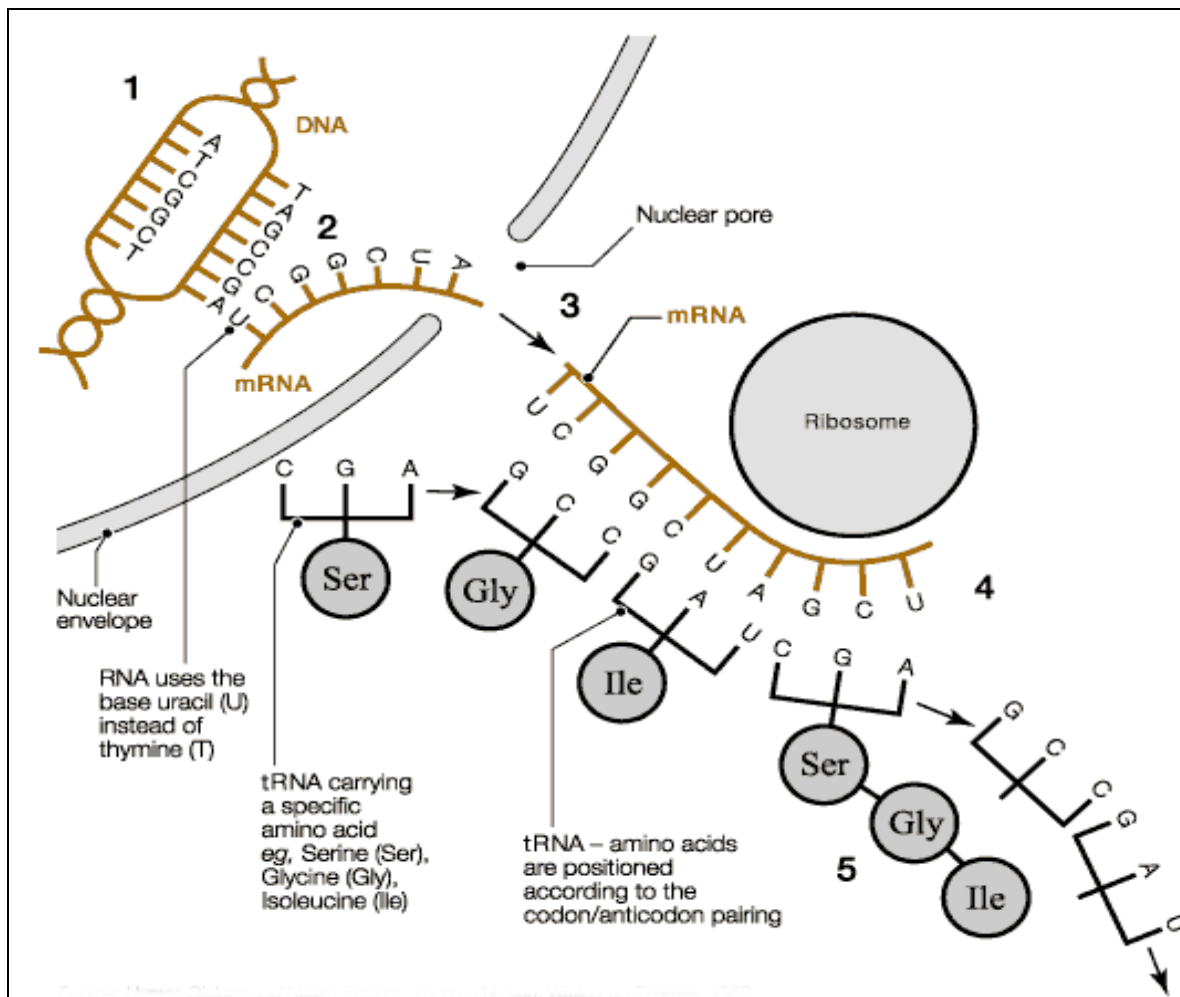


Figure 1: The principles of protein synthesis: 1. The DNA double helix unwinds to expose a sequence of nitrogenous bases. 2. A copy of one of the strands is made in a process known as transcription. The copy is made of messenger ribonucleic acid (mRNA) which, following transcription, travels out of the nucleus into the main body of the cell, where protein synthesis occurs. 3. The mRNA couples with the ribosome. Transfer RNA (tRNA) brings free amino acids to the ribosome. 4. The anticodon present on the tRNA recognises the codon present on the mRNA, and the ribosome adds the amino acid to the growing chain of linked amino acids (polypeptides), cleaving it away from the tRNA. This process is known as translation. 5. As the polypeptide chain grows, it folds to form a protein. (Givens and Reiss, 1996)

The measurements of RNA/DNA ratio were first carried out for fish larvae (Clemmesen, 1993), fish and other aquatic animals. Vrede *et al.* (2002) carried out measurements with *Daphnia* and

the method has been widely used in aquatic sciences. Edsman *et al.* (1994) ran measurements of RNA contents of signal crayfish and published some interesting results. Edsman *et al.* found strict correlations between the animals' RNA content and their nutritional status. Starvation immediately led to a decrease in RNA concentrations and the animals responded rapidly to refeeding. However, as mentioned above, crayfish have not earlier been target objects for the RNA/DNA ratio method as described above. They pose an extra challenge for measurements because of the high enzymatic activity in their tissue. Soon after a crayfish dies, the enzyme-induced decomposing starts and tissue samples disintegrate so fast that measurements of RNA and DNA make no sense anymore after the samples have been left at room temperature for more than 0.5 hours. Therefore, sampling and handling of the biological material have to be carried out in a clean and cooled environment and samples have to be kept refrigerated or frozen during the whole time of processing.

2.4.4 Experiments

To achieve the objectives described before, a number of different experiments were undertaken to cover the main issues. This study contains five different experiments each of which will be presented in a separate chapter.

The following specific problems were used to select and design experiments for this study:

1. An optimised diet composition concerning the ratios of proteins, lipids and carbohydrates will have to be examined. The diet will have to meet the macronutritional requirements of crayfish to provide optimal growth and survival. For this purpose, a response surface experiment was carried out. It was based on mixture approach methodology. Detailed description of this experiment is given in *Chapter 4*.
2. Effects of different types of diet on animals' growth and body composition will have to be examined to improve feeding control and minimize feed loss (*Chapter 5*).
3. How can growth in response to different treatments be assessed in short-term experiments? The introduction of new methodology in crayfish research (*Chapter 6*).
4. Different binding agents will have to be tested as well as their influence on bioavailability of essential nutrients (*Chapter 7*).

5. The effects of important additives such as carotenoids, vitamins and minerals as well as attractants to improve the acceptance of the diets will have to be studied to improve health and survival of the animals. (*Chapter 8*).

The results of this study should help farmers and researchers by providing a new method for the examination of physiological responses and by giving advice to develop suitable feeds for an intensified culture of crayfish.

With the time and resource available for this work, only a small area of all questions related to crayfish aquaculture and crayfish nutrition could be covered. Focus had to be restricted on a few main questions. The understanding of suitable macronutrient composition and type of crayfish diets was one of the main tasks of this study. Yet, more detailed work will be necessary after this study and crayfish nutrition will still remain an open field for research. The aim of this work was to provide a baseline of research, to open new opportunities and to provide new information about some major questions concerning crayfish aquaculture. This also included the question of how to assess crayfish growth and nutritional status reliably.

3 Materials and methods

The experiments of this study were carried out in the Evo Fisheries Research Station (Finnish Games and Fisheries Research Institute) located in southern Finland about 150 km northeast of Helsinki. The experimental tank system was purpose-built for this study and was situated in one of the station's research buildings.

Experiments were carried out from the beginning of 2001 until the late spring of 2003.

Table 1 gives an overview about each of the experiments and the main methods used.

Experiment	Tanks used	Methods	Replicates
Mixture approach	16	Growth, Protein, Lipid	1 to 3
Effects of diet types	12	Growth, Protein, Lipid	4
RNA/DNA ratio as a new method	16	Growth, RNA/DNA ratio	4
Effects of different binding agents	16	Growth, RNA/DNA ratio	4
Effects of attractants	15	Growth	5

Table 1: Experiments and detailed information about tanks methods and replicates used for this study. Growth method includes recording of wet weight gain, carapace length increment and dry weight. Protein was measured using the micro-Kjeldahl method; lipids were measured using sulphophospho-vanillin. Measurements of RNA/DNA ratio were carried out using the ethidium bromide method. The number of replicates refers to the number of tanks for each treatment.

3.1 Tank system

The experiments were carried out in a purpose-built system consisting of a total of 32 tanks.

The number of tanks used varied from 12 to 16 in different experiments.

The tanks had a surface area of 0.48m² (0.6m x 0.8m) and a height of 40cm. The floor of the tanks was covered with gravel and limestone (0.5cm - 1.0cm in diameter) and animals were provided shelter by adding plastic tubes as hideouts. Their length was 4.5cm with a diameter of 2.1cm. 40 shelters were provided for 20 animals. In addition, every tank was equipped with a plastic-coated wire frame reaching out of the water to allow the animals to climb above the water surface (Savolainen *et al*, 2003).

The water level in the tanks was adjusted to 12cm – 15cm by lowering or raising the outlet pipes. The outlets had a diameter of 32mm and were covered by metal filters with a mesh size of 1.5mm to prevent food loss and animal escapes.

Inlets of all tanks were situated at the opposite end of the outlets and water was exchanged with a constant rate of approx. 2l/min.

Flow rates and the exchange of water in this semi-open recirculated system were kept constant and the circulated water was treated by mechanical and biological filtration. The system was run with an average exchange of 250l per day and it had a total volume of 4000l. The ground water used in the system was pumped from the station's own well and the water temperature was kept in the range of 17.5 – 19°C with the help of a 5kW electric heater.

Steel meshes were used for mechanical filtration and a biofilter with a total volume of 200l and a filtration rate of 5l/min was connected to the system (Schneider and Wolf, 1998). *Figure 2* shows an overview of the system components. More details about the tanks and the system are available in *Pictures 2, 3 and 4*.

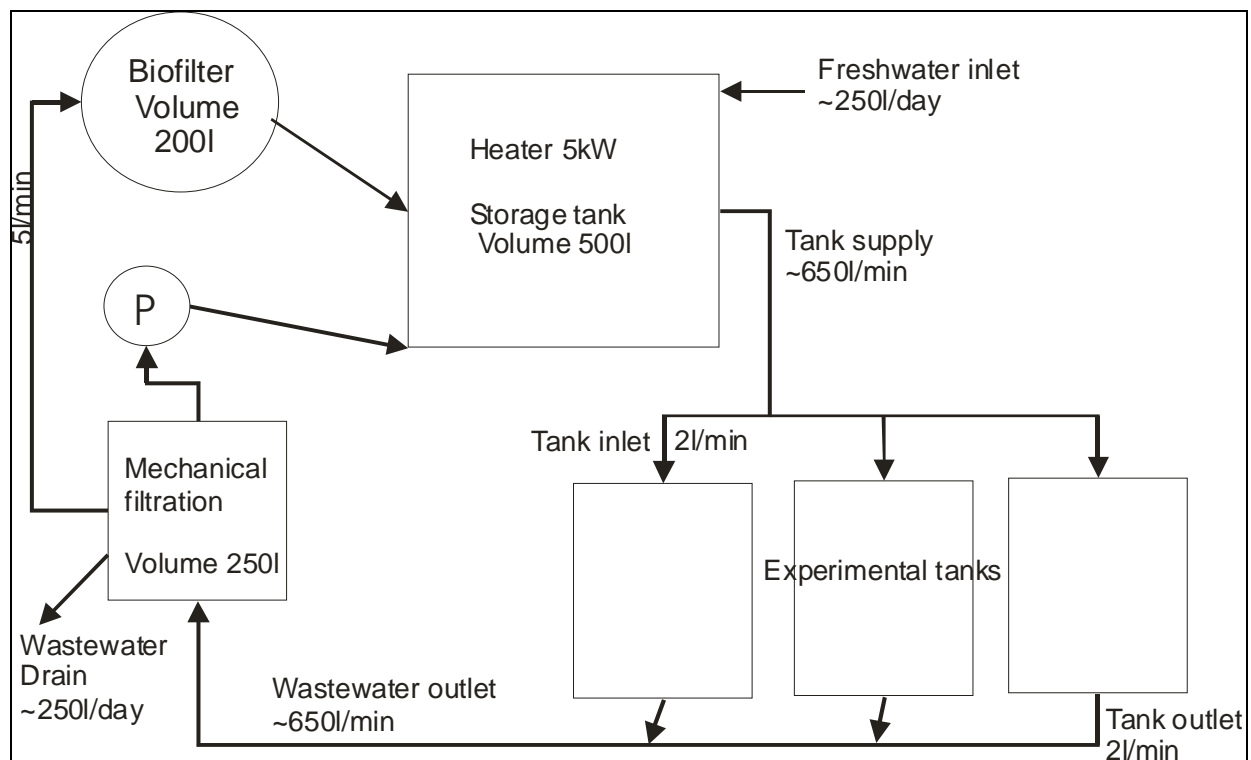


Figure 2: Schematic drawing of the system used for the experiments of this study. The entire system consisted of 32 tanks, the total water volume was 4000l. More detailed information are given in the text and in Pictures 2, 3 and 4.



Picture 2: View of the experimental tank system as it was used for this study. The system was artificially illuminated (18L:6D photoperiod) and temperature was kept within a range of 17.5 to 19°C.



Picture 3 (left): Photography showing one of the tanks (0,6m x 0.8m) in the experimental system. A substrate from gravel was provided and shelters were given in excess (40 shelters for 20 animals).



Picture 4 (right): Photography showing the shelters and the metal mesh added to each tank. On the upper end of the picture, the outlet can be seen. A small meshed filter was used to ensure, animals could not escape through the outlet pipes.

3.2 Experimental animals

Juvenile signal crayfish *Pacifastacus leniusculus* that were hatched either in the Evo Fisheries Research station or in the crayfish hatchery in Olkiluoto in western Finland were used in this study. Juvenile animals were used to ensure that the animals will undergo several moulting cycles during the experimental periods. Animals between 1 and 1.5 years old and with a carapace length of 13 to 26mm were chosen for the experiments. Before the experiments the animals were kept in outdoor ponds or fibreglass tanks and fed with a formulated diet supplied by Rehuraisio Ltd, a Finnish fish and crustacean feed company. The water to the outdoor ponds was taken from river Majajoki.

After the transfer to the experimental system, the animals were given at least 10 days for acclimatisation. For all experiments stocking-density was 40 animals per square metre equalling 20 individuals in each tank. During acclimatisation food was given daily except the day before the start of an experiment. The experiments were started by weighing and measuring the animals and ensuring there were 20 individuals in each tank.

3.3 Measurements, sampling and data analysis

Disturbance during the experimental period was reduced to a minimum. At the end of each experiment all animals collected, measured and weighed from each tank.

The weight of the animals was taken as grams to an accuracy of 0.01g using a Sartorius analytical A 120S digital laboratory balance (Sartorius AG, Göttingen, Germany). The carapace length of the animals was taken in mm (with an accuracy of 0.01mm) using a digital calliper (Biltema Ltd, Finland).

Growth data were calculated from the measured weights and carapace lengths. First, mean values of these were calculated for each tank and then growth in weight and length was calculated from the corresponding tank means.

After length and weight measurements all animals, tank-by-tank, were killed by freezing and taken for further analysis including dry weight, protein and lipid contents (Forster, 1970) and RNA/DNA ratio if applicable.

3.3.1 Lipid content measurement using the sulphospho-vanillin method

3.3.1.1 Reagents

Chloroform reagent

100ml of chloroform were mixed with 50ml of methanol.

Concentrated sulphuric acid

Colour reagent: phosphovanillin

1.978g of vanillin were dissolved in 56ml of distilled water and filled up to 1000ml with 944ml of phosphoric acid H₃PO₄.

A standard of 1mg/ml lipids was produced by mixing 10mg of olive oil with 10ml of chloroform reagent. For calibration this standard was diluted with chloroform reagent to measurable concentrations of 10, 25, 50, 75 and 100µg/ml.

3.3.1.2 Sampling and measurement

30mg of the dried sample were washed with 3ml of chloroform reagent and centrifuged for 10min at 4000rpm. The supernatant was withdrawn by a pipette and the sample was washed in the same way for additional two times.

0.5ml of the supernatant was then taken into a test tube and dried for one hour at 50°C; 0.5ml of chloroform reagent was used as a blank. After drying, 0.5ml of sulphuric acid was added to the sample. The tube was sealed with aluminium foil and boiled in exactly 100°C for 10min.

Immediately after heating the sample was cooled down to room temperature. 0.1ml of the sample was then taken into a new test tube and 2.5ml of vanillin reagent was added. The sample was left for 60min at room temperature before the absorption was measured in a UV/VIS spectrophotometer at 520 nm wavelengths. 10mm cuvettes were used for the measurement.

3.3.1.3 Calculations

The amount of lipids in the sample was calculated by using the function of the calibration curve.

The original lipid contents was calculated as follows:

$$C_{\text{tot}} = C_{\text{samp}} * V_{\text{sup}} / W_{\text{tot}}$$

C_{tot} = lipid concentration of the original sample ($\mu\text{g}/\text{mg dw}$)

C_{samp} = lipid concentration actually measured from 0.1ml ($\mu\text{g}/0.1\text{ml}$)

V_{sup} = total volume of the supernatant after washing the original sample (ml)

W_{tot} = original dry weight of the sample (mg)

The results of this measurement were calculated to μg lipids per mg wet weight using the wet weight/dry weight ratio measured earlier.

3.3.2 Determination of protein content using the micro-Kjeldahl-method

Determination of nitrogen

Samples were digested using sulphuric acid. The amino-nitrogen present was converted into ammonium, which was then measured photometrically using a phenol hypochlorite reaction.

3.3.2.1 Reagents

Digestion reagent

300mg of selenium dioxide were dissolved in 15ml of distilled water and mixed with 85ml of nitrogen free sulphuric acid. This solution was made up to 500ml with distilled water.

Phenol reagent

5ml of 80% phenol and 20mg of sodium nitroprusside were made up to 500ml with distilled water.

Hypochlorite reagent

2ml of sodium hypochlorite were added to 80ml of 2.5% NaOH and made up to 200ml with distilled water.

3.3.2.2 Sampling and measurement

Approximately 10mg of sample (1:10 dilution with silica gel) were heated with 1ml of digestion mixture at 120°C for 12 hours. The sample then was heated for another 6 hours at 320°C. The mixture was left for cooling down to room temperature and then diluted to 50ml with distilled water. 2ml of 2.5% NaOH, 4ml of phenol reagent and 2ml of hypochlorite reagent were added to 1ml of the diluted sample. The mixture was made up to 10ml with distilled water and left for 20 minutes at room temperature to allow development of the colouring. The samples were measured at 635nm in a 10mm cuvette.

Standards

Standards were prepared by dissolving 0.3820g of ammonium chlorite and a drop of chloroform in 1000ml of distilled water. This standard was diluted 1:10 (volumetric dilution) to a stock solution with a concentration of 10µg ammonium per ml. The stock solution was then used to produce standards of 50ml containing 0.5, 1.0, 2.0, 2.5, 3.33, 4.0, 5.0, 6.66 and 7.5 µg / ml.

Blank

A blank was prepared with 2% diluted digestion mixture.

3.3.2.3 Calculations

A calibration curve of the standards versus their absorbance values was drawn. A linear regression was calculated for this curve. The equation was used to calculate the amount of ammonium chlorite in the samples. These figures were multiplied by the dilution factor of 50 and divided by their sample weights to give the amount of ammonium chlorite X per mg sample.

From these figures, the weight of the nitrogen N in the samples was calculated using the molecular weights of ammonium chlorite:

$$X / 53.5 * 28 = N (\mu\text{g} / \text{mg})$$

These values were corrected for protein using the standard conversion factor of 6.25

$$N * 6.25 = \mu\text{g protein} / \text{mg}$$

The conversion factor is based on the work of Danish chemist Johan Kjeldahl. He discovered that "all protein" contains about the same amount of nitrogen (16%). He analysed for nitrogen, which is relatively easy, and calculated crude protein on the basis: $100/16 = 6.25$, therefore:

$$\text{NITROGEN} \times 6.25 = \text{CRUDE PROTEIN.}$$

3.3.3 RNA / DNA ratio measurement

For a few experiments additional measurements were carried out to see whether RNA/DNA ratio of crayfish muscle tissue can be used to compare the animals state of growth in regard to different treatments.

For these measurements a modified ethidium bromide method (Clemmesen, 1993) was used.

3.3.3.1 Reagents

Tris EDTA buffer

6.057g of Tris(hydroxymethyl)-aminomethane (Merck 8382), 5.844g of sodium chloride (Merck 6404) and 3.722g of Titriplex III (Merck 8418) were mixed and made up to 1000ml with distilled water. The solution was adjusted to a pH of 8.00 with concentrated HCl and kept in the refrigerator at 2-6°C.

Tris EDTA-SDS buffer

6.057g of Tris(hydroxymethyl)-aminomethane (Merck 8382), 5.844g of sodium chloride (Merck 6404), 2.00g of sodium dodecyl sulfate SDS (Merck 13760) and 3.722g of Titriplex III (Merck 8418) were mixed and made up to 1000ml. The solution was adjusted to pH 8.00 with conc. HCl and kept refrigerated at 2-6°C.

Tris NaCl buffer (RNase buffer)

1.1214g of Tris(hydroxymethyl)-aminomethane (Merck 8382) and 0.8766g of sodium chlorite were made up to 1000ml with distilled water. The solution's pH was adjusted to 7.5 with conc. HCl and the solution was kept refrigerated at 2-6°C.

Ethidium bromide reagent

10mg of ethidium bromide (Serva 21238) was dissolved in 100ml of distilled water. The working solution was kept in a covered Erlenmeyer flask and refrigerated at 2-6°C.

RNAse

A stock solution was prepared by dissolving 1.00mg of RNAse powder (Serva Ribonuclease A, 34388) in 1.00ml of Tris- NaCl buffer (pH 7.5). The solution was heated to 100°C for 15 min to destroy possibly present DNAses. After cooling to room temperature the solution was split into aliquots and stored at -20°C.

A working solution was prepared by adding 200µl of the stock solution to 1200µl of Tris-NaCl buffer.

DNA standard

For DNA standards Lambda DNA (Boehringer Mannheim, 745782) was used. This DNA standard comes in liquid solution of 0.25µg/µl. This solution was diluted 1:10 with Tris-EDTA buffer. Aliquots were stored in Eppendorf vials at 2-6°C.

RNA standard

16S,23S ribosomal RNA (Boehringer Mannheim, 206936) was used for preparing RNA standard. The solution containing 4.00µg/µl was diluted 1:10 with Tris-EDTA buffer, split into aliquots and stored in Eppendorf vials at -20°C.

3.3.3.2 Sampling

The RNA/DNA ratio was measured using muscle tissue from the abdominal muscles of individual crayfish. Prior to the measurements the animals were killed by freezing them at -35°C.

The preparation of the samples took place using a cold store with room temperature not exceeding 5°C.

Sampling was carried out by removing the exoskeleton from the frozen animals and taking tissue samples of 250mg from the abdominal muscle. The samples were weighed to an accuracy of 5mg and kept in Eppendorf vials for further processing. 800µl of ice-cold Tris-EDTA-SDS buffer and 2 spatulas of different size glass balls were then added and the samples were homogenised using a Retsch swinging mill (15 min, 10000rpm). The homogenised samples

were then centrifuged for 8 min at 6000rpm. 500µl of the supernatant was pipetted and split into two aliquots of 250µl. The aliquots were stored for measurement at -20°C.

3.3.3.3 Measurement

The content of total nucleic acid (RNA + DNA) was measured first and the content of DNA was determined after RNase had digested RNA in the sample. The fluorescence was read by using a spectrophotometer, with 365 nm excitation and 590 nm for emission.

First, one aliquot of the samples was made up to 1150µl with Tris-EDTA buffer and was then measured without ethidium bromide for determination of the sample's self-fluorescence.

Further, 115µl of ethidium bromide solution were diluted with 1150µl of Tris-EDTA buffer and measured to determine the fluorescence of the reagent. The sample was then mixed with 115µl of ethidium bromide to measure the total nucleic acids contents. The second aliquot was made up to 900µl with Tris-EDTA buffer and was then mixed with 250µl of the RNase working solution. The sample was then left 20 min at 30°C for digestion of RNA. After measuring the sample's self-fluorescence the sample was mixed with 115µl of ethidium bromide and measured as described.

3.3.3.4 Calculation

A calibration curve resulting from measurements of dilutions of DNA and RNA standards was drawn and the equation for the linear regression was calculated. The equation was used for further calculation of the samples' DNA and RNA contents.

The measured self-fluorescence of the samples and the ethidium bromide solution were first subtracted from the fluorescence measured. The real fluorescence was then used to calculate the nucleic acid contents of the samples.

After RNase treatment, the DNA contents of the samples was calculated from the corrected fluorescence values using the equation calculated from the DNA calibration curve. By including the dilution factors, the total contents of DNA in µg in the samples was calculated.

The fluorescence measured for DNA was then subtracted from the corrected total fluorescence of nucleic acids. The resulting fluorescence represented the RNA present in the samples. With the help of the equation calculated from the RNA calibration curve and the dilution factor the RNA contents in μg of the samples was calculated.

Finally, the RNA/DNA ratio was calculated using the total amounts of RNA and DNA in the samples.

$$CF_{(RNA+DNA)} = TF_{(RNA+DNA)} - SF_{(EB)} - SF_{(Sample)}$$

$$CF_{(DNA)} = TF_{(DNA)} - SF_{(EB)} - SF_{(Sample)}$$

$$CF_{(RNA)} = CF_{(RNA+DNA)} - CF_{(DNA)}$$

$CF_{(RNA+DNA)}$ = corrected fluorescence of the sample without RNase treatment

$TF_{(RNA+DNA)}$ = total fluorescence of the sample without RNase treatment

$SF_{(EB)}$ = self fluorescence of ethidium bromide

$SF_{(Sample)}$ = self fluorescence of the sample

$CF_{(DNA)}$ = corrected fluorescence of the sample after RNase treatment

$TF_{(DNA)}$ = total fluorescence of the sample after RNase treatment

$CF_{(RNA)}$ = calculated fluorescence of the RNA in the sample

3.4 Diets

The diets used in the experiments for this study were all purpose-made to meet the macronutritional requirements in all other aspects than those under investigation (Ackefors *et al.*, 1992, Jussila 1997). Diets were prepared from raw ingredients like fishmeal, wheat-starch, and fish oil. The different concentrations of these main components resulted in varying dietary compositions of the feeds. Fishmeal was chosen to serve as a source for proteins, fish oil for lipids and wheat-starch for carbohydrates. A presentation of the nutritional compositions of the raw ingredients is given in *Table 2*.

Nutrient	Raw ingredient		
	Fishmeal	Fish oil	Wheat-starch
Protein %	70.5	0	2.4
Lipid %	9.3	99	0.2
Carbohydrates and fibres %	0	0	89.8
Water %	9.5	1	7
Ash %	10.7	0	0.6

Table 2: Macronutritional composition of the raw ingredients used to prepare the diets used in the experiments. All values are given as percentage. Data refer to measurements carried out by Ruohonen and Vielma (1994). Micronutrients were not taken into account.

Minerals, vitamins, choline and phospholipids like lecithin formed a base mix that was added to supply the animals with all additional nutrients necessary. Although the concentrations of the different macronutrient ingredients were changed in regard to the experimental set-up, the amount of base mix used was kept constant to ensure a sufficient supply of vitamins and minerals.

Carotenoids in the form of astaxanthin were added to the diets to support good pigmentation of the animals' exoskeleton as well as their physiological functions.

Minerals play a very important role in moulting and the hardening of the exoskeleton and vitamins had to be added to ensure the animals were not suffering from any malnutrition.

Phospholipids like lecithin are types of lipids that are needed by every living cell in an animal. Cell membranes as well as the protective sheath surrounding the brain are composed mostly of lecithin and it is also found in the muscles and nerve cells.

Choline is the major precursor of betaine, and it is used to produce the important neurotransmitter acetylcholine. It assists in nerve impulse transmission, liver functions and lecithin production.

The base mix was always prepared prior to the preparation of each batch of food and the fresh mix was then added to the raw ingredients at the time of preparation. To avoid vitamin-choline interaction, choline was added to the diets separately.

Each diet was prepared in the same way: 1.00kg of the dry ingredients according to the formulation were blended and then mixed with 350 ml of distilled water to form a dough that was then cold-pelletised with a household food processor (Kenwood Ltd) and left drying at a constant temperature of 30°C for 48 hours in a laboratory drying oven (Termacks, Ltd). The

dried pellets were kept in sealed bags in a refrigerator at 5°C for a maximum of 4-5 weeks. During the time of storage, no fouling or disintegration of the diets could be observed. Feeding of the animals depended on the experiment's schedule and will be described in detail in connection to each experiment in the oncoming chapters. In general, the amount of diet presented to the animals at each feeding was kept constant during the full period of each experiment. The total amount of daily fed diet as fed did not exceed 5% of the animals' initial bodyweight.

All tanks were cleaned on regular basis and uneaten food was removed daily. Exuviae of moulted animals were removed immediately to avoid providing a supplementary nutritional source. Animals that died during the experimental period were also removed and calculations were always based on the actual number of animals being alive on the time of measurement.

3.5 Statistical analysis

Statistical analyses for the results of the experiments were carried out using the “R” language (The R Foundation for Statistical Computing, 2003). Growth in terms of wet weight and carapace length, protein contents and lipid contents as well as dry matter contents were used to judge the response of crayfish to the various treatments. Wet weight gain and carapace length growth were expressed as growth in g and mm, respectively, and calculated according to $w_t - w_0$ where w_t and w_0 are the final and initial wet weight or carapace length. Growth data were mostly analysed using general linear models. The general form of the model was as follows:

$$Y = p_0 + p_1X_1 + p_2X_2 + \dots + p_nX_n + \epsilon$$

Y = Response variable

$p_0, p_1 \dots p_n$ = Estimated parameters

$X_1, X_2 \dots X_n$ = Predictor variables

ϵ = Residuals

The best statistical model describing the data for each response was chosen on the basis of lack of fit tests, incremental parameters and the extra sums of squares principle (F-test). All models were fitted and tested with the "R" language (The R Foundation for Statistical Computing, 2003) and their adequacy checked by studying the model residuals.

The mixture components (fishmeal, fish oil and wheat-starch) used in the experiment described in *Chapter 4* were transformed to corresponding pseudocomponents and the responses modelled with linear, quadratic or super-cubic polynomials specific for mixture designs (Cornell, 1990).

The models were as follows:

$$\text{Linear: } Y = p_1x_1 + p_2x_2 + p_3x_3 + \epsilon$$

$$\text{Quadratic: } Y = p_1x_1 + p_2x_2 + p_3x_3 + p_{12}x_1x_2 + p_{13}x_1x_3 + p_{23}x_2x_3 + \epsilon$$

$$\text{Super-cubic: } Y = p_1x_1 + p_2x_2 + p_3x_3 + p_{12}x_1x_2 + p_{13}x_1x_3 + p_{23}x_2x_3 + p_{123}x_1x_2x_3 + \epsilon$$

Y = Response variable

x_1 = Fishmeal pseudocomponent

x_2 = Fish oil pseudocomponent

x_3 = Wheat-starch pseudocomponent

$p_1 \dots p_{123}$ = Estimated parameters

ϵ = Residual error

Again, the best statistical model describing the data for each response was chosen on the basis of lack of fit tests, incremental parameters and the extra sums of squares principle (F-test) and the models were fitted and tested with the "R" language (The R Foundation for Statistical Computing, 2003) including their adequacy checks by studying the model residuals. The mixture models are presented graphically as triangular plots. A triangular plot consists of three variables with a constant sum. The constant sum constraint means that there are just two independent pieces of information (Ruohonen *et al.*, 2003). By this, it is possible to plot observations in two dimensions within a triangle. *Figure 3* explains how to read the plots.

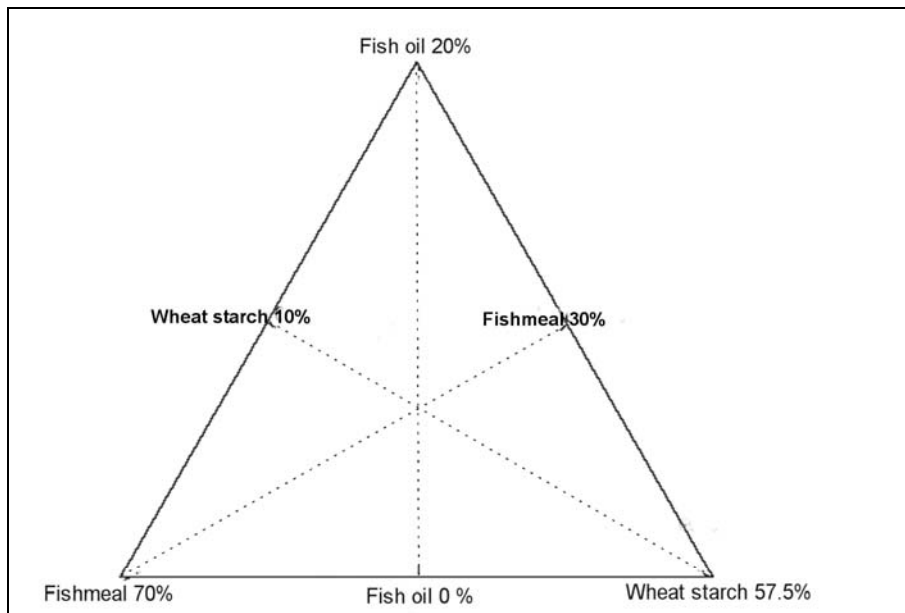


Figure 3: Reading the components of a triangular plot. The level of a component is read perpendicular to the component axis (dashed lines) (Ruohonen et al., 2003, modified).

The diagnostics to check the adequacy of statistical models were based on the model residuals.

Fig. 4 shows an example of the typical model diagnostics undertaken for the linear model analysis of carapace length increment in the experiment “RNA/DNA ratio as an instrument of growth measurement for juvenile signal crayfish, *Pacifastacus leniusculus*” presented in Chapter 6. The diagnostics include residuals against the fitted values. A (standardised) Residual vs. Fitted Value plot helps to detect behaviour not modelled in the underlying relationship, i.e. lacking predictors, and to detect heterogeneous variance.

In the second diagnostic plot, a visual check for the fit of a theoretical distribution to the observed data by examining the *quantile-quantile* (or Q-Q) plot is possible. In this plot, the observed residuals of a model are plotted against the theoretical quantiles. A good fit of the theoretical distribution to the observed values would be indicated by this plot if the plotted values fall on a straight line.

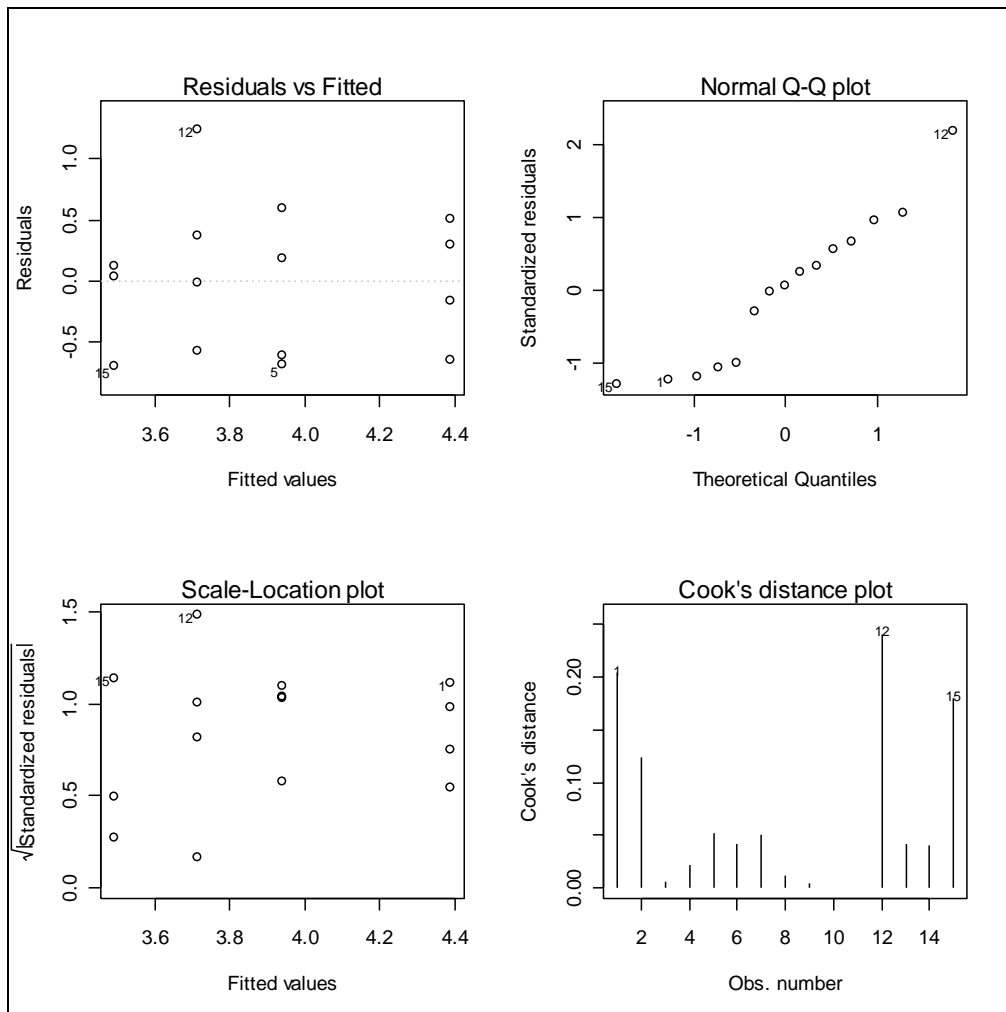


Figure 4: Model diagnostics from the “R” language. The diagnostics include: a plot of residuals against fitted values, a Scale-Location plot of $\sqrt{| residuals |}$ against fitted values, a Normal Q-Q plot, and a plot of Cook's distances versus row labels (Obs number = observation number in the data).

A “Scale-Location” plot (S-L) of $\sqrt{| residuals |}$ against fitted values is also included in the diagnostics. The S-L plot has the square roots of the absolute value minus their group medians on the vertical axis and the group medians on the horizontal axis.

The basic idea can be applied to other measures of location and spread. That is, some measure of spread (or variability) is plotted on the vertical axis and some measure of location is plotted on the horizontal axis. This plot is useful for looking for outliers and checking the assumption of constant variance (The Plot page, 2004).

The “Scale-Location” plot, also called “Spread-Location” or “S-L” plot, takes the square root of the absolute residuals in order to diminish skewness.

This S-L and the Q-Q plot use standardised residuals which have identical variance (under the hypothesis).

The last diagnostic tool is Cook's distance plot. This plot is useful for identifying potentially influential points. Cook's distance is another measure of impact of the respective case on the regression equation. It indicates the difference between the computed values and the values one would have obtained had the respective case been excluded. All distances should be of about equal magnitude; if not, then there is a reason to believe that the respective case(s) biased the estimation of the regression coefficients (R Documentation, 2004).

For certain designs with replicates at the levels of the predictor variables, the residual sum of squares can be further partitioned into meaningful parts which are relevant for testing hypotheses. Specifically, the residual sums of squares can be partitioned into lack-of-fit and pure-error components. This involves determining the part of the residual sum of squares that can be predicted by including additional terms for the predictor variables in the model (for example, higher-order polynomial or interaction terms), and the part of the residual sum of squares that cannot be predicted by any additional terms (i.e., the sum of squares for pure error). A test of lack-of-fit for the model without the additional terms can then be performed, using the mean square pure error as the error term. This provides a more sensitive test of model fit, because the effects of the additional higher-order terms are removed from the error.

4 Effect of different diet compositions on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus*

4.1 Introduction

Increased knowledge of crayfish nutrition is the key to successful aquaculture intensification. Basic diets for freshwater crayfish provided by feed companies like Rehuraisio Ltd. enable farmers to grow crayfish in extensive aquaculture already today. However, growth and survival of the animals are not good enough to intensify crayfish farming and most farmers cannot rely on artificial diets without supplements. Often the diets available are only given as supplementary feed to crayfish farmed in extensive outdoor ponds. Making crayfish farming more competitive requires an intensification of farming methods and thus, an increase in diet quality. Improvements in the composition of crayfish diets can only be reached by increasing the understanding of the animals' macronutritional requirements and feeding biology. The composition of crayfish diets has to take into consideration the essential needs of freshwater crustaceans. Therefore, aspects like the amounts of minerals, vitamins and essential supplements like carotenoids play a very important role in this process. However, the first task is to find suitable macronutrient (protein, lipid, carbohydrate) compositions. Therefore, in this experiment, the focus was drawn on the main components of a suitable crayfish diet.

In their natural habitats crayfish occupy low trophic levels and they feed on aquatic vegetation, benthic invertebrates and associated detritus as well as zoo- and phytoplankton. These omnivorous feeding habits are one of the key factors for the successful establishment of crayfish culture systems.

Crayfish have been described as "uniquely polytrophic" animals and may be regarded as herbivores, predators and detritivores. Their mode of feeding displays preferences for certain types of diets but also show acceptance of various diets in accordance with age, season of the year and physiological status (Goddard, 1988). Evidence gained from field and laboratory

studies shows that juvenile crayfish feed mostly on aquatic invertebrate prey and adult crayfish feed more on vegetation and detritus. The development of suitable diets is therefore an important issue in crayfish culture research.

4.2 Experimental set-up

4.2.1 Mixture approach

In this experiment, the effect of 8 different dietary compositions on growth and body composition of juvenile signal crayfish *Pacifastacus leniusculus* was studied.

The type of experiment for this kind of question is called a mixture experiment (Ruohonen, 1998). The experimental region is a triangle, also called a simplex, and all factors are constrained by the fact that their sum must be 1 or 100% (*Fig. 5*), i.e.

$$X_1 + X_2 + X_3 = 1$$

X_1 = Fishmeal component

X_2 = Fish oil component

X_3 = Wheat-starch component

This design is a special case of the so-called response surface methodology (RSM), and the answer to the problem is a fitted response surface over the experimental triangle that is capable to prediction within the experimental region, see Cornell (1990). The response surface is fitted with regression methods.

The advantages of the mixture design are as follows:

- No need to use fillers; we work with actual raw ingredients for practical diets.
- What you design is what you get, i.e. effects are not confounded with any unintentional parallel changes of factors.
- Results are readily applicable to the feed industry because the experimental and application spaces are the same.

(Ruohonen and Kettunen, 2004)

As the diets used in this experiment all contain an unchanged level of base mix (12.5%) with vitamins, minerals and binder, the amounts of the essential components cannot sum up to 1, but to 0.875. Therefore, the calculations are carried out with the help of pseudocomponents

$$x_1 + x_2 + x_3 = 1$$

neglecting the base mix.

x_1 = Fishmeal pseudocomponent

x_2 = Fish oil pseudocomponent

x_3 = Wheat-starch pseudocomponent

Pseudocomponents, in effect, rescale the constrained data area so the minimum allowable amount of each component is zero and the sum of all components under investigation is 1. This makes a constrained design in pseudocomponents the same as an unconstrained design in proportions.

The formulation of the different diets and their equivalent pseudocomponents are presented in *Table 3*.

The tested compositions were chosen according to D-optimality criteria for optimal experimental design (e.g. Ruohonen and Kettunen, 2004).

Diet	Fishmeal X_1	Fishmeal ps x_1	Fish oil X_2	Fish oil ps x_2	Starch X_3	Starch ps x_3	Sum Components	Sum Pseudocomponents
1	0.3000	0.3429	0.0000	0.0000	0.5750	0.6571	0.8750	1.0000
2	0.3000	0.3429	0.0984	0.1125	0.4766	0.5447	0.8750	1.0000
3	0.3000	0.3429	0.2000	0.2286	0.3750	0.4286	0.8750	1.0000
4	0.4695	0.5366	0.0986	0.1127	0.3069	0.3507	0.8750	1.0000
5	0.5063	0.5786	0.0000	0.0000	0.3687	0.4214	0.8750	1.0000
6	0.5750	0.6571	0.2000	0.2286	0.1000	0.1143	0.8750	1.0000
7	0.7000	0.8000	0.0000	0.0000	0.1750	0.2000	0.8750	1.0000
8	0.7000	0.8000	0.0750	0.0857	0.1000	0.1143	0.8750	1.0000

Table 3: Effect of diet composition on growth and body composition. Components X_1 , X_2 , X_3 and pseudocomponents ps x_1 , x_2 , x_3 of all different diets used in the experiment. The components sum up to $X_1 + X_2 + X_3 = 0.875$ without the base mix. The pseudocomponents correct this $x_1 + x_2 + x_3 = 1$.

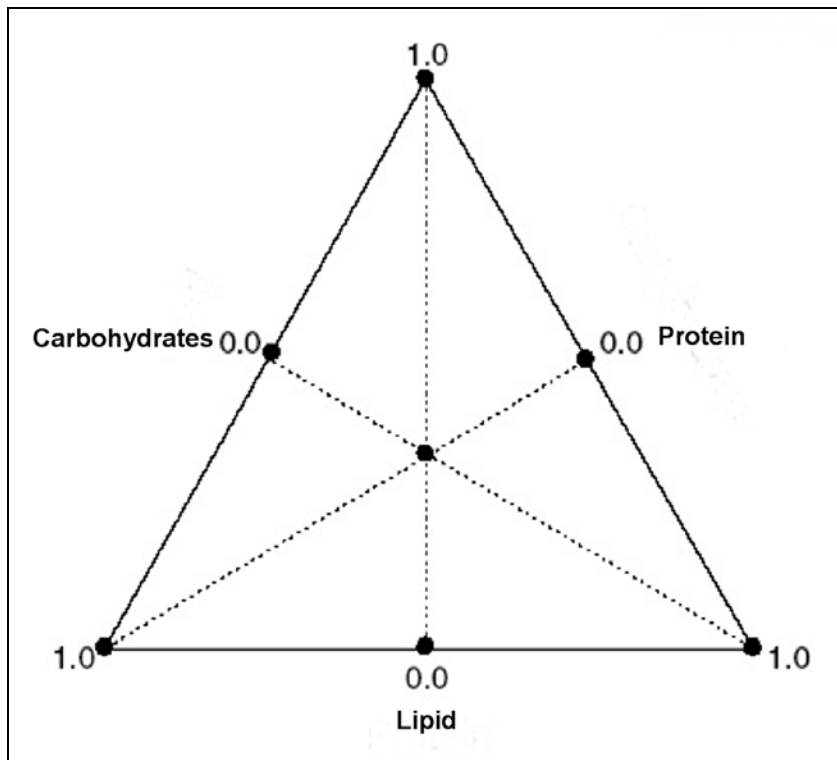


Figure 5: Effect of diet composition on growth and body composition. Experimental region of an unconstrained mixture design is a triangle. Dots show possible optimal runs. (Ruohonen and Kettunen, 2004, modified)

4.2.2 Diets

The diets were all prepared from the same ingredients that were used in varying amounts. This leads to different contents of proteins, lipids and carbohydrates in the diets. The basic ingredients for the diets used in this experiment were fishmeal as a source for protein, fish oil as a lipid source and wheat-starch for carbohydrates.

Concentrations of fish oil reached from 0% to 20%, the contents of fishmeal reached from 30% to 70% and starch was added to concentrations reaching from 10% to 57.5%. *Pictures 5 and 6* show two of the diets used in this trial.



Picture 5 (left): Detailed photography of a diet used in this comparative experiment. The diet in the picture (left) contains 20% fish oil, 30% fishmeal and 37.5% starch, resulting in a softer texture of the pellets after drying.

Picture 6 (right): Detailed photography of a diet used in this comparative experiment. The diet showed in the picture contains 70% fishmeal, 10% starch and no fish oil. The pellets are very firmly textured.

In addition to the main components a basic mixture (base mix) of vitamins, minerals, carotenoids and supplements as lecithin, choline and cholesterol were added to all diets in equal amounts. Further, a cellulose-based binding agent “Finnfix” was added to the base mix in a concentration of 2.0% DW.

Carotenoids were added to the diets to support good pigmentation of the animals’ exoskeleton. Minerals play a very important role in moulting and the hardening of the exoskeleton and vitamins had to be added to ensure the animals were not suffering from any malnutrition.

Phospholipids like lecithin are type of lipids that are needed by every living cell in an animal. Cell membranes as well as the protective sheath surrounding the brain are composed mostly of lecithin and it is also found in the muscles and nerve cells.

Choline is the major precursor of betaine, and it is used to produce the important neurotransmitter acetylcholine. It assists in nerve impulse transmission, liver functions and lecithin production.

The base mix was prepared just prior to the preparation of the diets and added to the raw ingredients at the time of preparation. The amount of the base mix was 12.5% of all diets.

All diets were based on a formulation of 1000g of ingredients. The exact formulation and the resulting calculated proximate composition of each diet is given in *Table 4*.

Fishmeal %	Fish oil %	Wheat-starch %	Protein %	Lipid %	Carbohydrates and fibre %	Water %	Ash %
30.00	0.00	57.50	22.53	2.91	51.64	6.88	3.56
30.00	9.84	47.66	22.29	12.63	42.80	6.28	3.50
30.00	20.00	37.50	22.05	22.67	33.68	5.68	3.44
46.95	9.86	30.69	33.84	14.19	27.56	6.71	5.21
50.63	0.00	36.87	36.58	4.78	33.11	7.39	5.64
57.50	20.00	10.00	40.78	25.17	8.98	6.36	6.21
70.00	0.00	17.50	49.77	6.55	15.72	7.88	7.60
70.00	7.50	10.00	49.59	13.96	8.98	7.43	7.55

Table 4: Effect of diet composition on growth and body composition. Detailed formulation and resulting composition of the different diets used in this mixture experiment. All data are given in %. The amount of base mix added to the raw ingredients remained unchanged.

Each diet was prepared in the same way: 1000g of the dry ingredients were blended and then mixed with 350ml of distilled water to form a dough that was then cold-pelletised with a household food processor (Kenwood Ltd) and left drying at a constant temperature of 30°C for 48 hours in a laboratory drying oven (Termacks Ltd). The dried pellets were kept in sealed bags in a refrigerator at 5°C for a maximum of 4-5 weeks. During the time of storage, no fouling or disintegration of the diets could be observed.

4.2.3 Handling and measurements

Before the experiment was started, animals were given a time of 2 weeks for acclimatisation.

The experiment was started by weighing and measuring the animals.

The tank system has already been described in detail in *Chapter 3 “Material and methods”*. For this experiment, 16 tanks were randomly chosen and stocked with 20 crayfish each. Every tank was equipped with shelters in excess and the initial stocking density was 40 animals/m².

Dead animals as well as exuvia and other remains of food or animals were removed from the tanks daily and the controlled environment of the experimental system was used to create optimal temperature and light conditions for the animals. Water temperature was kept within the range from 17.5 to 19°C and an 18L:6D photoperiod was generated by artificial illumination.

The animals were fed daily except weekends and the amount of food given each day was kept constant during the 4-month experiment. The amount of diet given to the animals was calculated to represent 5% of the total body mass of all animals in one tank in the beginning. The animals were measured and weighed at the beginning and the end of the experiment to collect information about the animals' growth during the experimental period.

At the end of the experiment, all animals were killed by deep-freezing and stored at –25°C for further analysis. The analysis carried out contained the determination of lipids using the sulphophospho-vanillin method and proteins using micro-Kjeldahl method, as well as the animals dry weight by freeze-drying. The exact procedures of these measurements are described in detail in *Chapter 3 “Material and methods”*. The measurements were carried out using pooled samples of all tanks and running the measurements with 5 replicates of each sample. Results measured were then pooled to calculate the mean values for each tank.

4.2.4 Statistical analysis

Wet weight and carapace length growth, protein contents and lipid contents as well as dry matter contents were used to judge the response of crayfish to the various diets. Wet weight and carapace length growth were expressed as growth in mm or g and calculated according to $w_t - w_0$ where w_t and w_0 are the final and initial wet weight or carapace length. Tank means were kept separate for statistical analysis. The mixture components (fishmeal, fish oil and wheat-

starch) were transformed to corresponding pseudocomponents and the responses modelled with linear, quadratic or super-cubic polynomials (Cornell, 1990). These models were as follows:

$$\text{Linear: } Y = p_1x_1 + p_2x_2 + p_3x_3 + \epsilon$$

$$\text{Quadratic: } Y = p_1x_1 + p_2x_2 + p_3x_3 + p_{12}x_1x_2 + p_{13}x_1x_3 + p_{23}x_2x_3 + \epsilon$$

$$\text{Super-cubic: } Y = p_1x_1 + p_2x_2 + p_3x_3 + p_{12}x_1x_2 + p_{13}x_1x_3 + p_{23}x_2x_3 + p_{123}x_1x_2x_3 + \epsilon$$

Y is the response variable, x_1 is the fishmeal pseudocomponent, x_2 is the fish oil pseudocomponent, x_3 is the wheat-starch pseudocomponent, $p_1 \dots p_{123}$ are estimated parameters and ϵ is the residual error (Ruohonen *et al.*, 2003). Detailed explanation about the methods and the resulting plots are given in *Chapter 3 "Materials and methods"*.

4.3 Results

Initial carapace length varied from 12.53mm to 23.47mm, the animals' initial wet weight reached from 0.4g to 3.43g. Mean carapace length was 16.14mm and the mean wet weight was 1,08g

At the end of the 4-month experiment the final carapace length reached from 16.13mm to 35.74mm with a mean of 25.44mm. Wet weights at the end of the experiment varied from 1.27g to 11.28g with a mean wet weight of 4.73g.

Mean values for each treatment group were obtained by calculating them on the basis of tank means. The resulting means are given in *Table 5*.

Diet	Length increment	Weight		Protein		Lipids		
	(mm)	SD.	gain (g)	SD.	(mg/g ww)	SD.	(mg/g ww)	SD.
1	9.56	0.22	3.80	0.24	122.75	3.2	20.1	0.93
2	8.90	NA	3.57	NA	111.07	NA	35.79	NA
3	8.11	0.26	3.17	0.1	124.25	1.96	37.32	8.09
4	9.78	0.08	3.8	0.32	118.88	26.79	31.63	12.93
5	9.51	0.11	3.93	0.58	124.17	34.91	18.52	0.08
6	9.28	0.13	3.21	0.53	121.91	1.5	34.84	9.74
7	9.31	0.42	3.77	0.74	117.25	15.93	19.66	4.08
8	9.99	0.12	3.98	0.09	120.78	5.54	31.32	5.64

Table 5: Effect of diet composition on growth and body composition. Mean values and standard deviations for all measurements in relation to the different diets used in this comparative experiment. N = 2 for all treatment groups except N = 1 for Diet 2 (SD not available (NA)) and N = 3 for Diet 4.

4.3.1 Growth data

Wet weight gain was calculated from tank means in initial and final wet weight. Wet weight gain varied from 2.99g to 4.06g. Different weight gains were obtained from the different treatment groups. A detailed presentation of all data measured and calculated for the different treatment groups is given in *Table 5*.

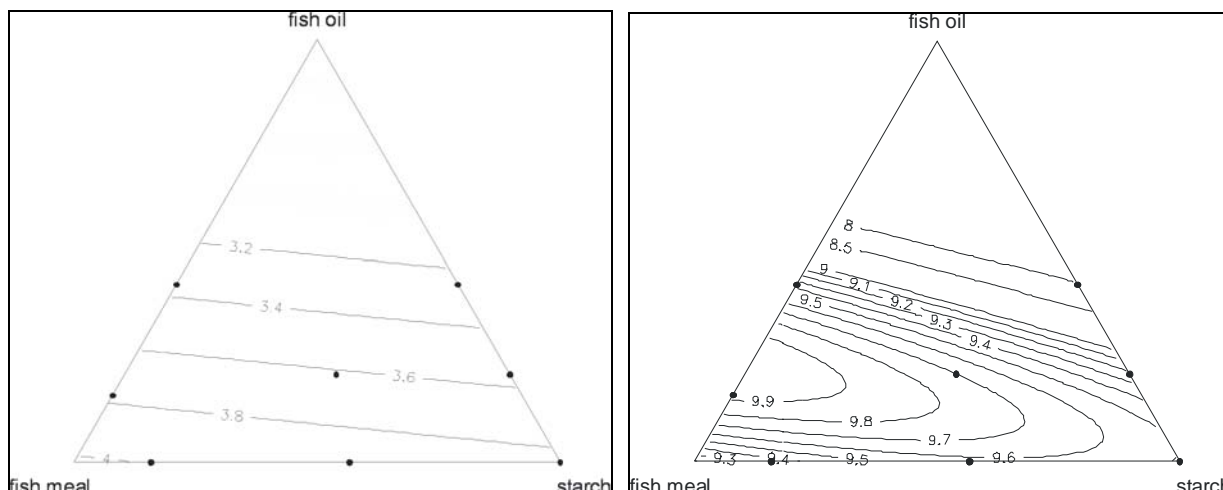


Figure 6 (left): Weight gain responses (in g) in relation to experimental mixtures of fishmeal, fish oil and wheat-starch (starch). Fitted response contours are restricted to the experimental region. The dots stand for each treatment.

Figure 7 (right): Carapace length increment responses (in mm) in relation to experimental mixtures of fishmeal, fish oil and wheat-starch (starch). The dots stand for each treatment. Isobars represent the increase in carapace length as measured in mm. Fitted response contours are restricted to the experimental region

In terms of wet weight growth the different diet compositions turned out to have statistically significant effects on the outcome of the experiment. Lack of fit (LOF) test revealed that linear model was suitable for the analysis of the wet weight gain. For the pseudocomponents the following estimates were calculated:

	Estimate	SE
Fish oil ps	2.44	0.30
Fishmeal ps	4.02	0.13
Starch ps	3.85	0.12

Table 6: Effect of diet composition on growth and body composition. Resulting estimates and standard errors from the statistical analysis of wet weight increase in the response surface experiment. Calculations are based on tank means from the experiment. Calculated results refer to pseudocomponents (ps).

For wet weight increment a residual standard error of 0.23 on 13 degrees of freedom was measured. Linear model revealed significant influence of dietary components on wet weight gain ($p = 2.2e-16$, $F = 1345$). The model is presented graphically in Fig. 6.

Carapace length increased during the period of the experiment. Growth varied from 8.05mm to 10.06mm. The average growth in terms of carapace length increase differed between the treatment groups. Details are given in *Table 5*.

Statistics on the measurements of carapace length increment were carried out and LOF test and model diagnostics suggested the quadratic model to be the best suitable model describing the data (*Figure 7*). The model fitted the data well with $p = 0.0229$ and $F = 4.9769$. The residual standard error was 1.4344 with 8 degrees of freedom.

4.3.2 Protein and lipid contents

The mean body protein contents of various treatment groups varied from 94.83mg/g ww to 148.85mg/g ww and details are given in *Table 5*.

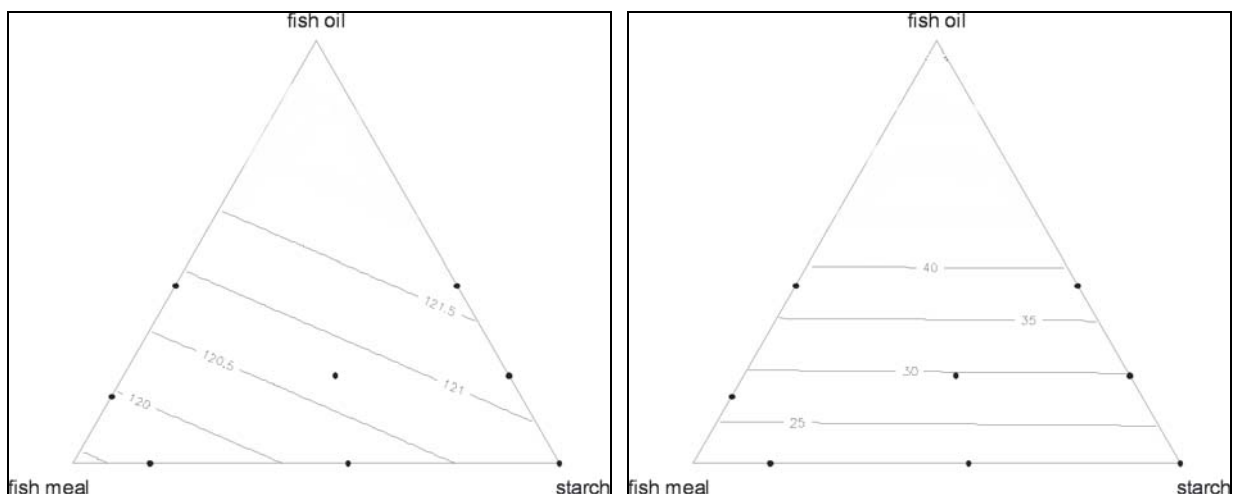


Figure 8 (left): Effect of diet composition on growth and body composition. Protein content responses (in mg/g ww) in relation to experimental mixtures of fishmeal, fish oil and wheat-starch (starch). Fitted response contours are restricted to the experimental region. The dots represent the actual experimental diet compositions. Isobars represent the fitted values of the statistical model that was estimated on the basis of the measurement values.

Figure 9 (right): Effect of diet composition on growth and body composition. Responses in lipid content (in mg/g ww) in relation to experimental mixtures of fishmeal, fish oil and wheat-starch (starch). Fitted response contours are restricted to the experimental region. The dots stand for the experimental diet compositions, the isobars the fitted values of the statistical model that was estimated on the basis of the measurement values.

The best statistical model among the three mixture models (see above) describing the effects of macronutrient components on body protein content appeared to be the linear one. Estimates and standard errors (SE) of the model parameters are given in *Table 7*.

	Estimate	SE
Fishmeal FM ps	119.407	8.647
Fish oil FO ps	122.895	19.890
Starch ST ps	120.829	8.160

Table 7: Effect of diet composition on growth and body composition. Resulting estimates and standard errors from the statistical analysis of tank based protein contents data from the experiment. The estimates refer to pseudocomponents (ps).

Statistics for the protein content data showed a significant effect of the components on the protein content of the animals. The best model was quadratic with $p = 1.92e-12$ and $F = 321.7$ with 3 and 13 degrees of freedom. Residual standard error was 15.53 with 13 degrees of freedom. Even though statistics revealed an effect of the various diet compositions on body protein, this effect seems to be fairly small. Over the experimental range in *Figure 8* the protein content changes roughly 2mg/g ww out of 120mg/g ww.

The mean lipid contents of the body on different experimental diets are given in *Table 5*.

Body lipid content reached from 16.77mg/g ww to 46.40mg/g ww.

The simplest adequate model for lipid contents was the linear model. Estimates and standard errors are given in *Table 8*. Statistics showed a significant influence of the diet on the body lipid content of the animals ($p = 6.834e-09$ and $F = 88.12$ with 3 and 13 degrees of freedom). The residual standard error was 7.17 with 13 degrees of freedom.

Over the range of the experimental diet composition, changes in the animals' body lipid were far more obvious than the changes in protein content. Increased lipid levels in the diet compositions led to increased body lipids. The results suggest a relationship between dietary lipid content and the body lipids of crayfish.

	Estimate	SE
Fishmeal FM ps	21.025	3.992
Fish oil FO ps	61.670	9.183
Starch ST ps	21.424	3.767

Table 8: Effect of diet composition on growth and body composition. Resulting estimates and standard errors from the statistical analysis of tank based lipid content data from the experiment. Calculated results refer to pseudocomponents (ps).

4.4 Discussion

Using the mixture experiment principles and response surface methodology have been the keys to the success of this experiment. The methods chosen proved to be efficient tools to study the potential gross formulations of the major ingredients (fishmeal, fish oil and wheat-starch) for an improved crayfish diet. Due to the design of the experiment, no artificial fillers had to be used to obtain the experimental compositions. This led to the usage of real diets that are ready to use not only in experiments but also in aquaculture. The applicability of the feeds used make them similar to the products of the feed industry and shortens the time needed for the development of suitable diets for freshwater crayfish.

The mixture approach covers a wide range of ratios of the major food components, protein, lipids and carbohydrates. The experimental design helped to screen a variety of gross formulations in only one experiment. The number of diets (8) requires a high number of tanks making the experimental-set-up very complicated. The high number of tanks used for the experiment is a considerable weakness of the method but results support the feasibility of the mixture approach and it clearly outweighs classical one-variable-at-time (OVAT) experiments. One other benefit of the mixture methodology is the avoidance of unintentional parallel changes in other food components when one component is deliberately changed.

Statistically, parallel changes result in the inability to estimate and separate the effects of interest because they are confounded by the unintentional effects (Ruohonen and Kettunen, 2004).

Additionally, the design of the experiment allows monitoring the interactions of the macronutrients.

The examination of these influences allows a development of suitable diets and the improvements of dietary composition that are not possible with conventional OVAT methods.

Based on the results of this study, it can be stated that juvenile signal crayfish *Pacifastacus leniusculus*, grow best on diets containing between 33.5% and 45% protein, 9% to 33% carbohydrates and 5% to 14% lipid, i.e. fairly wide ranges appear to produce similar results. Best growth results were obtained with diets containing less than 10% lipid. Crayfish did not benefit from an increase in dietary protein from 34% to 49% when increasing the amount of fish oil simultaneously that increased the dietary energy level.

The findings are backed by experiences made by different researchers (Järvenpää, *pers. com.*) though others have found lower levels of protein to be sufficient. Protein requirements are

reported to be between 20% and 30% for noble crayfish (*A. astacus*) (Ackefors and Lindqvist, 1994) and red swamp crayfish (*P. clarkii*) (Huner and Meyers, 1979), while Huner and Lindqvist (1984) found, with diets containing 30% or 40% protein, that the higher level increased glycogen and lipids reserves in the hepatopancreas, which is seen as an indication of better condition. Jones *et al.* (1997) found that low protein levels (15% or less) resulted in slower growth and nutritional stress for yabbies (*Cherax destructor*).

Freshwater crayfish require low levels of lipids in their diets (D'Abramo and Conklin, 1985; Goddard, 1988; Fotedar *et al.* 1997), with indications of growth inhibition when the levels exceeded 8% (Andrews *et al.*, 1972; D'Abramo, 1979). The lipid content of 0.1% to 3% has been shown to be adequate for crayfish, if such sterols as cholesterol and fatty acids including highly unsaturated fatty acids like EPA and DHA are included (Zandee, 1966; D'Abramo *et al.*, 1997; Lee and Wickins, 1992).

Carbohydrate requirements of freshwater crayfish are unclear, but the crayfish readily utilise diets with high levels of carbohydrates and the commercial diets normally contain carbohydrate levels of 25% or more (Jussila, 1997). Common carbohydrate sources in crayfish diets are starch and other soluble polysaccharides, which are used as an alternative source of energy to protein (D'Abramo *et al.*, 1997).

Differences in dietary energy content are insufficient to explain the equally good growth performance with low-protein and high-protein diets in this study. Better growth was achieved by increasing the proportion of starch at the expense of fish oil, thus keeping the fishmeal contents of the feed constant. In addition to differences in the energy value between fish oil and starch, metabolic disturbance due to the load of lipid in fish oil may partially explain these results (D'Abramo *et al.*, 1997, Lee and Wickins, 1992). In their natural environment, crayfish feed on plants, detritus and carrion. None of the natural diets contain high levels of lipids. It is therefore likely that increased lipid contents of the artificial diets lead to inhibited growth rates.

In addition to wet weight growth and carapace length increment, protein and lipid content measurements provide information on the suitability of diets differing greatly in their gross composition. Protein contents measured from crayfish were dependent on the dietary composition although the effect was small. High levels of protein resulted in higher protein concentrations in the animals' tissue. At high fishmeal levels, better protein concentrations were achieved by increasing the proportion of starch at the expense of fish oil. A clear tendency of

increased lipid deposition with higher dietary fish oil content was apparent at the expenses of body protein.

This tendency should also be taken into account when examining the growth data, since an increase in body lipid at the expenses of body protein seems to have inhibiting influence on the animals' growth. Unlike fish, crayfish do not show a real capability to utilise high lipid levels in their diets. The relationship between body lipid and protein content occurs to be unregulated. As the experimental outcome has shown, crayfish can handle higher loads of lipid in presence of high protein levels. With decreasing protein levels, lipid seems to become more and more inhibiting factor for growth. The relationship between body lipid and body protein contents shows unusual patterns in comparison to fish (Ruohonen *et al.*, 2003) and will certainly have to be examined in further experiments.

On a growth basis, fish oil was a clearly more inhibiting factor than an enhancing feedstuff, most probably due to the missing regulation capacity of the animals. The higher energy content of fish oil did not result in any enhanced growth. In the light of the present data, high body protein contents can be achieved with diets that are capable of supporting very different growth rates depending on the non-protein source selected. Good protein economy was achieved with low-fish oil, high-wheat-starch diets, but these diets supported poorer growth and were less efficient than high-fishmeal diets.

Crayfish are polytrophic animals and this nutritional flexibility is the basis of several commercially successful farming methods (Huner and Lindqvist, 1995). Furthermore, different species have different dietary preferences when choices of aquatic plants or higher aquatic organisms (Covich, 1977; Warner *et al.*, 1995) are available. The nutrition in some of the extensive or semi-intensive pond culture systems relies on detritus based ecosystems that are created by growing plants in the ponds (Huner, *pers. com.*). The focus of future research should therefore include the possible replacement of animal products in crayfish diets. The replacement of fish oil with suitable vegetable fat e.g. may increase the digestibility (Järvenpää, *pers. com.*) and food acceptance due to closer resemblance with natural food sources.

5 Effects of different types of diet on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus*

5.1 Introduction

In this study the effects of different types of diets given to juvenile crayfish were examined. Aggressive interaction between individuals as well as food stability can have major influence on the animals' growth and survival (Ahvenharju, *pers. com.*, Savolainen *et al.*, 2003). Crayfish in populations and in aquaculture systems seem to follow clear hierarchies (Ahvenharju, *pers. com.*, *unpublished data.*). The interactions between the animals at high stocking densities may lead to increased mortality and injuries and lower growth rates (Savolainen *et al.*, 2004). Attempts to reduce the interactions between individuals have been made by providing shelter and hiding places (Savolainen *et al.*, 2003) as well as removing faster growing large animals (Ahvenharju, *pers. com.*, *unpublished data.*). Fighting for food can lead to severe injuries and increased mortality as well as increased growth of dominant animals while sub-ordinant individuals may have disadvantages and suffer from lack of nutrients (Holdich, 2001). Additionally, observations have shown that the animals carry food to their shelters to "own" their food. This leads to a deterioration of the shelter environments by decomposing feeds. Observations in the Olkiluoto Crayfish Hatchery (Järvenpää, *pers. com.*) have suggested that aggressive interactions between individuals fighting for food might be avoided by offering the diets in large portions. It was hypothesised for this study that these portions should have a size and weight that makes it impossible for individuals to carry the diet away and several animals were supposed to be feeding on one unit at a time. Furthermore, it was anticipated that larger blocks of food would have better water stability and thus longer availability in the water. This way of presenting the diet was thought to decrease aggressive behaviour and increase the feeding efficiency.

5.2 Experimental set-up

Preliminary laboratory tests suggested that larger portions of diet had a higher stability and were thus available to the animals for a longer period of time before they started disintegrating. In preliminary experiments blocks of diet weighing between 3.5g and 4.2g were stable in water for

2.5 to 3 hours while pelletised diets of the same type started disintegrating already after 1.5 hours. These preliminary experiments were carried out by adding the diets to 200ml of distilled water at room temperature (20°C) and slowly stirring the samples with a magnetic stirrer. The samples were observed every 10 min and the measurement was stopped when the diets showed clear signs of disintegration by falling apart or dissolving in the water.

The actual experiment was carried out using 12 tanks of the experimental system and randomly dividing these tanks into three treatment groups of 4 tanks each. Every tank was stocked with 20 animals each resulting in a stocking density of 40 animals/m². Tanks were equipped with shelters in excess (40 shelters for 20 animals) and a metal mesh frame provided climbing possibilities for the animals. The system was run in semi-open recirculation and the temperature was kept within a range of 18.5-19°C. All animals were given 2 weeks time for acclimatisation before the experiment was started and initial weight and carapace length were measured and recorded.

For this experiment, three different kinds of diets were designed. Two groups of animals were fed an artificial diet that was composed of wheat-starch, fishmeal, fish oil and a basic mix containing minerals, vitamins and carotenoids as well as Finnfix as a binding agent.

The following formulations were used to prepare the diets for this experiment:

wheat-starch (31.0%), fishmeal (54.0%) and fish oil (2.5%) as well as a base mix (12.5%) containing vitamins, minerals, carotenoids and Finnfix (2.0%) as a binding agent.

The formulation of the raw ingredients resulted in a final calculated composition of 38.8% protein, 7.6% fat, 27.8% carbohydrates and fibre and 6.0% ash. The water content was 7.3%.

All values are on as-fed basis. The compositions were calculated using the nutritional values of the raw ingredients given by Ruohonen and Vielma (1994). Details are given in *Chapter 3 "Materials and methods", Table 2.*

Mixing ingredients with distilled water formed a dough and processing it with a Kenwood household food processor produced the basic diet that was then air-dried at 30°C for 48h in a laboratory drying oven.

The first treatment group was fed with a pelletised diet. Pellets were about 3-3.5mm in diameter with a length of 5-9mm.

The second treatment group was given the same diet but shaped as blocks of 3.5cm by 5cm. The thickness of the blocks was 4mm. Samples of the diets can be seen in *Pictures 7 and 8.*



Picture 7 (left): Detailed photography showing the block shaped artificial diets used in this experiment.



Picture 8 (right): Photography showing the typical pelletised diet usually used in feeding trials. Both diet types consist of identical raw ingredients.

The third treatment group in this experiment was fed with pieces of frozen roach. This group was used as a control group to assess differences between the block-shaped diet and a natural food source. Frozen roaches were chopped to small pieces of 0.5cm to 1.0cm and portions of 3.5-4.5g and were fed to the animals daily. Proximate composition for roach was taken from Berg (1986): 17.5% protein, 3.0% fat, 4.5% ash and 75.0% water.

Feed was given daily except on weekends. The amount of food on as-fed basis slightly exceeded 5% of the initial body weight of all animals in one tank and was kept constant over the experimental period.

All tanks were thoroughly cleaned once a week and uneaten food as well as exuviae or dead animals were removed daily. At the beginning and at the end of the experiment the animals were weighed and measured to collect data on initial weight and length and to ensure there were no significant differences between the three treatment groups. Final measurements were carried out at the end of the experiment. The measurements included the usual collection of wet weight and carapace length data. In addition, the animals' dry weight was measured after freeze-drying and measurements of protein and lipid contents were carried out. For this purpose, all animals were killed by deep-freezing at the end of the 4-month experiment and further measurements were carried out in the laboratory. The laboratory measurements included measurement of dry weight, determination of lipid content and determination of protein content. The dry weight of all individuals was determined by freeze-drying the animals for 48h at -50°C. The individuals

were numbered to compare the animals' wet and dry weight and to calculate the dry matter content. A mean for each tank was then calculated.

The freeze-dried animals were then pooled on the basis of their experimental tank and minced in a sample mill before further measurements were carried out.

The lipid content of the samples was determined by using the sulphophospho-vanillin method. A detailed description of the method can be found in *Chapter 3 "Materials and methods"*.

The determination of protein in the samples was carried out by usage of the micro-Kjeldahl method the description of which is also given in *Chapter 3 "Materials and methods"*.

Both measurements were carried out in the laboratory of the Evo Fisheries Research Station soon after the experiment was ended.

Statistical analysis of the results was carried out using the "R" language (The R Foundation for Statistical Computing, 2003). General linear models with the type of diet as treatment were used to analyse the data in this experiment.

5.3 Results

Mean values for the different measurements in relation to the treatments are given in *Table 9*.

Measurements	Diets			Measurements	Diets		
	Fish	Block	Pellet		Fish	Block	Pellet
Growth				Body composition			
Initial carapace length (mm)	20.70	20.56	20.50	Dry matter content (% ww)	31.43	31.58	31.53
SD	0.54	0.25	0.28	SD	0.62	0.49	0.27
Skewness	-0.49	1.22	0.28	Skewness	-0.01	1.24	1.80
Kurtosis	0.75	2.00	-0.65	Kurtosis	-4.16	0.71	3.23
Initial wet weight (g)	2.48	2.45	2.45	Protein content (mg/g ww)	138.12	119.99	118.68
SD	0.24	0.07	0.09	SD	3.37	4.57	8.21
Skewness	-1.18	-1.89	0.57	Skewness	-0.47	0.71	1.88
Kurtosis	2.29	3.60	-2.77	Kurtosis	-1.44	-1.26	3.60
Final carapace length (mm)	26.71	27.58	27.34	Lipid content (mg/g ww)	26.55	31.71	31.24
SD	1.08	0.68	0.68	SD	1.31	1.01	1.01
Skewness	-1.43	-0.11	-0.38	Skewness	-0.16	0.21	-1.98
Kurtosis	2.66	-5.34	1.49	Kurtosis	-4.73	1.52	3.93
Final wet weight (g)	5.44	6.13	5.91				
SD	0.19	0.17	0.15				
Skewness	-1.98	-0.47	-1.88				
Kurtosis	3.94	-1.95	3.53				
Increase in cl (mm)	6.01	7.03	6.85				
SD	0.71	0.56	0.89				
Skewness	-0.12	0.52	-0.98				
Kurtosis	-1.35	-3.07	1.57				
Increase in ww (g)	2.96	3.68	3.47				
SD	0.46	0.42	0.53				
Skewness	-1.62	-0.23	-1.74				
Kurtosis	2.51	-2.74	2.95				

Table 9: Effect of different types of diets on growth. Mean values, standard deviations skewness and kurtosis for the measurements carried out in this experiment. The values represent the means of different treatment groups. For all treatments $N = 4$, ($N = 20$ for lipid and protein content measurement).

Initial carapace lengths varied from 20.00mm to 21.30mm and wet weight from 2.14g to 2.74g. Wet weight at the end of the experiment varied from 4.43g to 6.61g and carapace length from 25.17mm to 28.17mm.

Carapace length increased from 5.17mm to 7.75mm with a mean of 6.46mm. The treatment-based results of the experiment are given in *Table 9*. Crayfish fed on fish grew 6.01mm, those fed on block feed 7.02mm and the animals fed the pelletised feed 6.84mm in length.

Statistical analyses did not reveal any significant influence of the diet type on the animals' carapace length increment ($p = 0.1649$, $F = 2.2169$). Taking the block shaped diet (7.03mm) as a baseline, analyses resulted in effect sizes of -1.02mm for crayfish fed on fish and -0.18mm for the animals fed with pellets. The residual standard error was 0.7313 with 9 degrees of freedom.

Calculated wet weight gain varied from 2.29g to 4.12g with a mean of 3.21g.

Animals fed with fish gained 2.96g ww, crayfish fed on the block diet gained 3.68g and those fed on the pellet diet increased their wet weight with 3.46g. *Table 9* provides a detailed listing of the treatment-related results.

Linear model analysis for the results of the experiment in terms of wet weight gain found no statistically significance for the effect of diet type on weight growth: $p = 0.1419$, $F = 2.4449$. The block shaped diet (3.68g) served as a baseline and analysis gave effect sizes of -0.78g for crayfish fed on fish and -0.22g for animals fed on pellets. The residual standard error was 0.4732 with 9 degrees of freedom.

The resulting dry weights differed from 1.42g to 2.09g with dry matter contents varying from 30.8 to 32.3%. A detailed listing of the treatment-based results is given in *Table 9*.

Linear model analysis did not show any significant effects of the treatments on the animals' dry matter contents: $p = 0.9115$ and $F = 0.09357$. Analysis returned effect sizes of -0.15% for crayfish fed on fed on fish and -0.05% for the animals fed with pellets. The baseline was 31.58% for the animals fed on blocks. The residual standard error was 0.48 on 9 degrees of freedom.

Results from the experiment suggest a trend of getting slower growth of the group fed on fish compared to the groups fed on the artificial diets although the effect did not reach statistical significance. The difference in the growth between the block fed group and the fish fed group was 21.2% in terms of wet weight and 14.5% in terms of carapace length, which are clearly biologically meaningful. It seems that although the number of replicates in this experiment was high, four, the statistical power of this wasn't sufficient.

Figures 10, 11 and 12 present graphically the results in relation to different treatments.

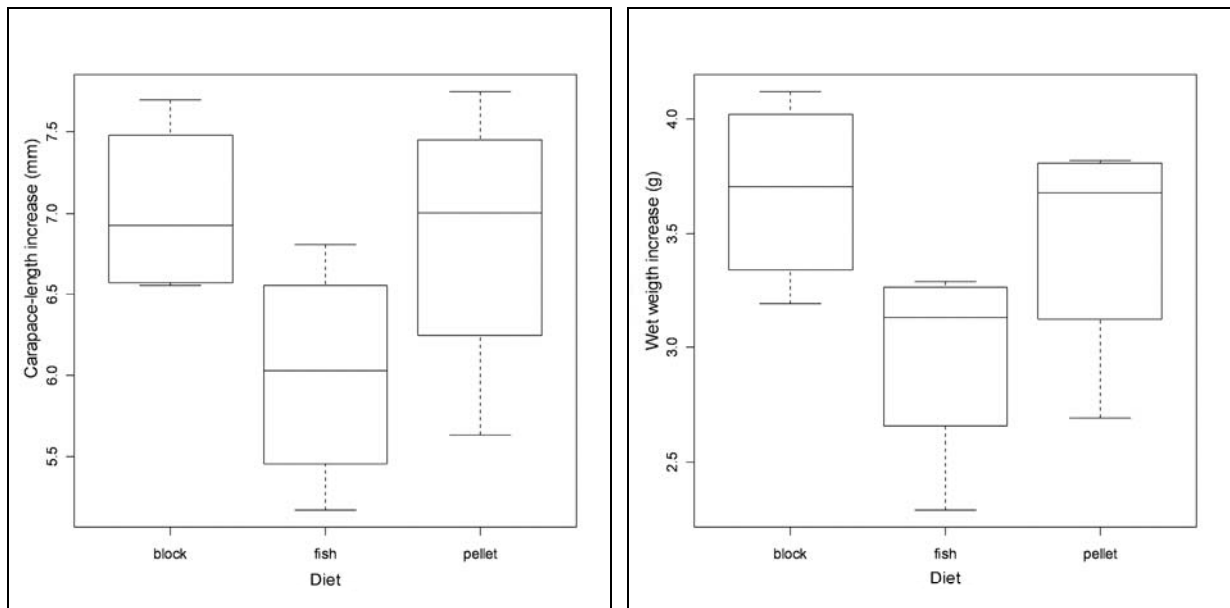


Figure 10 (left): Effect of different types of diets. The figure shows the relation between the different treatments (diets) and the outcome of the experiment in terms of carapace length increase. All specific values are given in Table 8 (means) or in the text. Data based on different treatment groups. For all treatments $N = 4$

Figure 11 (right): Effect of different types of diets. The figure shows the influences of the different diets wet weight increase of the animals. All specific values are given in Table 8 (means) or in the text. Data based on different treatment groups. For all treatments $N = 4$

Body protein contents ranged from 114.7 to 141.6mg/g wet weight and body lipids from 25.1 to 33.9mg/g ww.

Crayfish fed on fish had a low lipid content of 26.6mg/g wet weight and a protein content of 138.1mg/g wet weight. Animals fed with pellets showed a lipid content of 31.2mg/g wet weight and a protein content of 118.7mg/g wet weight, while animals fed on block diet had a lipid content of 31.7mg/g wet weight and a protein level of 120.0mg/g wet weight.

The differences between the treatment groups can be seen from Figures 13 and 14.

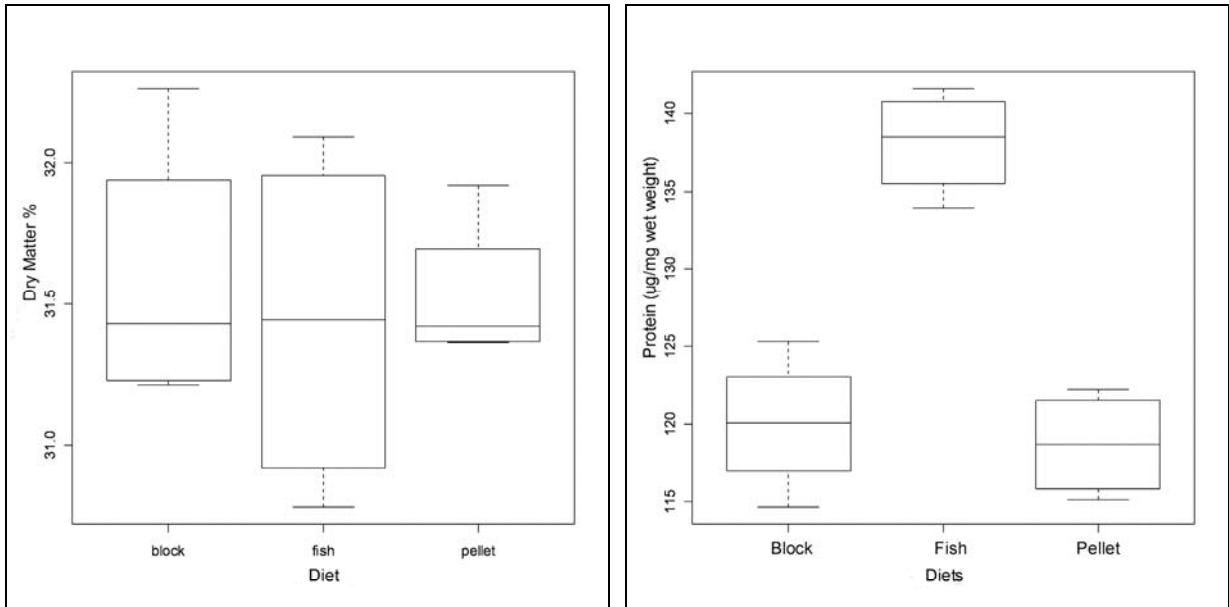


Figure 12 (left): Effect of different types of diets. The figure shows the relation between the different treatments (diets) and the outcome of the experiment in terms of dry matter. For all treatments $N = 4$.

Figure 13 (right): Effect of different types of diets. The figure shows the influences of the different diets on the final protein contents of the animals. For all treatments $N = 4$.

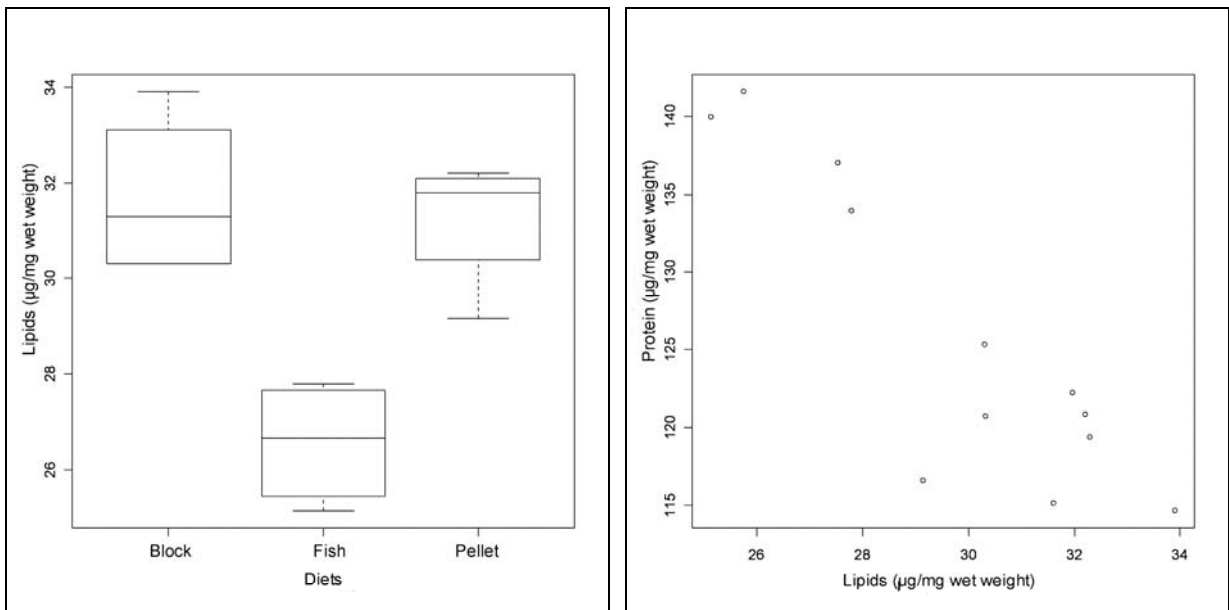


Figure 14 (left): Effect of different types of diets. Influences of the different diets on the final lipid contents of the animals. All specific values are given in Table 9 (means) or in the text. Data based on different treatment groups. For all treatments $N = 4$.

Figure 15 (right): Effect of different types of diets. Relationship between protein contents and lipid contents as measured in this experiment. All specific values are given in Table 9 (means) or in the text. Data based on different treatment groups. For all treatments $N = 4$.

As visible in Figures 13, 14 and 15, there was a significant difference measurable between the treatment groups when analyses of protein and lipids were carried out.

Statistics performed on the results of the lipid content showed a statistically significant difference between the crayfish that fed on block or pellet feed and those that fed on fish ($p = 0.0015$, $F = 14.49$). Effect sizes were -5.15mg/g ww for those fed on fish and -0.47mg/g ww for crayfish fed on pellet feed with block feed serving as a baseline (31.71mg/g ww). The residual standard error was 1.50 with 9 degrees of freedom.

The measurements of protein content differed in opposite direction. Protein content was clearly higher for crayfish fed on fish than for those fed on pellet and block feed. The mean values are given in *Table 9* and the effect was statistically significant ($p = 0.00006532$, $F = 33.8$). Effect sizes from the linear model were (baseline 119.99mg/g ww for crayfish fed on blocks) 18.13mg/g ww for those fed on fish and -1.31mg/g ww for animals fed on pellets. The residual standard error was 3.74 with 9 degrees of freedom.

The large differences in protein and lipid contents and the obvious relationship between protein and lipid content are also clearly visible in *Table 9* and *Figures 13, 14* and *15*. Specifically, there appeared a negative relationship of body protein and lipid content of crayfish even when they were expressed in relation to wet weight (*Figure 15*).

5.4 Discussion

Laboratory tests suggested that larger portions of diet had a higher stability and were thus available to the animals for a longer period of time before they started disintegrating. It was the aim of this study to investigate the effects of different diet types on growth of signal crayfish. The experiment produced similar growth results for animals either fed with blocks or pellets. Fish feeders showed a statistically insignificant lower growth rate in terms of weight gain and length increase. The dry matter contents of all animals were measured after freeze-drying them at the end of the experiment. Measurements of dry matter did not reveal any significant differences. Differences in all these growth measurements were small and statistically not significant. They might show some tendencies and it seems a diet only containing fish does not support good growth in a long-term experiment, while there were no real differences detected between pellets and blocks. Once more, it has to be stated that results might turn out differently in long-term experiments. A mid-term experimental period of 3 months seems to be insufficient to examine effects on weight gain and length increment due to the complicated nature of crayfish growth patterns.

To gain essential information about the different diets' effects, standard methods of measuring the body composition of freshwater crayfish were included in the experiment. The efficiency of digestion and absorption of crude protein has been measured in several studies (Forster, 1970), concluding that crude protein digestibilities are high. Animal sources of protein have been under investigation and found to be more digestible than vegetable sources (D'Abramo *et al.*, 1997), with crustaceans showing only small differences to domestic animals' capability of digesting different protein sources.

The measurement of lipids and proteins has been used to describe physiological responses of freshwater crayfish to changes in nutritional sources and different kinds of diet in the first part of this study. The methods used in this experiment are well established and widely used in physiological research. For crustaceans, however, limited studies have been conducted to evaluate the influence of dietary protein on protein content of body tissue (D'Abramo *et al.*, 1997). Measurements of lipid contents were carried out using the sulphophospho-vanillin method; protein content was measured using a modification of the micro-Kjeldahl method. Lipid contents turned out to be different between the group fed with fish and the groups fed the artificial diets. The lipid contents of pellet feeders and block feeders were 31.2 and 31.7µg/mg wet weight respectively. Animals fed with fish had a lipid content of 26.6µg/mg wet weight. Measurements of protein contents revealed an inverse trend in body protein levels. Fish feeders had the highest protein content (138.1µg/mg wet weight) while block feeders and pellet feeders contained a significantly lower amount of protein (block: 120.0µg/mg wet weight, pellets: 118.7µg/mg wet weight) and did not differ from each other significantly.

The huge differences in protein and lipid levels can only result from the different nutritional composition of the two diet types. Roach does not have a high lipid content (3%) and can hardly function as a source of lipids. On the other hand, the diet used in pellets and blocks had a lipid content of 7.3%. In addition, the carbohydrate content of the diet showed huge differences. Roach does not contain any carbohydrates; the artificial diets contained 27.8%. Carbohydrate requirements of freshwater crayfish are somewhat unclear, but the crayfish readily utilise diets with high levels of carbohydrates (*Chapter 4*) and the commercial diets normally contain carbohydrate levels of 25% or more (D'Abramo *et al.*, 1997).

It has been stated earlier that crayfish require low levels of lipids in their diets (D'Abramo and Conklin, 1985; Goddard, 1988; Fotedar *et al.* 1997), with indications of growth inhibition when the levels exceeded 8% (Andrews *et al.*, 1972; D'Abramo, 1979). Following studies by several authors (Zandee, 1966; D'Abramo *et al.*, 1997; Lee and Wickins, 1992), a lipid content of 0.1 to

3% has is seen to be adequate for crayfish, if such sterols as cholesterol and fatty acids including highly unsaturated fatty acids like EPA and DHA are included.

The results of this study suggest that even a moderate lipid level of less than 8% can already have major influence on the animals' body composition. However, the results do not show any inhibitory effect of lipid in the offered concentrations. In addition, the influence of the different carbohydrate levels cannot be fully assessed, as necessary knowledge is not yet available.

It therefore seems that the results of this second approach to examine the levels of body protein and lipid support the hypothesis from the first experiment (*Chapter 4*). Crayfish apparently are not capable of actively balancing their body protein/lipid ratio and the composition of their diet has major influence on their body composition. The storage of lipids also appears to be dependent on the amount of lipids available through the diet. Crayfish generally are known to have low lipid contents. Feeding trials have earlier shown that crayfish favour low-lipid diets (D'Abramo *et al.*, 1997; Järvenpää, *pers. com.*, Claybrook, 1983) and the inhibitory effects of high lipid levels have been described by Goddard (1988) and Fotedar *et al.* (1997).

It is therefore likely that they lack an active regulation of their body lipid contents. As there are two broad patterns of response, conformation and regulation, the increased lipid contents of the animals fed with the artificial diet might well be a conformation reaction. This would also explain the changes in protein contents due to the higher amounts of lipids present in the artificial diets. Measurements carried out by Huner and Meyers (1979) suggest protein levels vary from 38% to 48% for different crayfish species. It can therefore not be ruled out, that major changes within one species will be caused by the animals' limited regulatory abilities. In their natural environment, signal crayfish feed omnivorous with a high amount of low lipid plants and algae included in their diet. A balanced diet of algae, detritus, plants and invertebrates (Holdich, 2001) does not raise the need for an ability to regulate their protein/lipid ratio. The ratio might therefore easily be influenced by major changes in nutritional sources. The typical linear reaction for such a conformation can be derived from the data of the present experiment. *Figure 16* shows the protein/lipid ratio for all tanks of the experiment. Different treatments are distinguishable by their significantly different protein/lipid ratio. The influences of diet composition on the body composition of freshwater crayfish will have to be examined in further studies. The aspects of protein/lipid ratio need more investigation and unless more experiments have been carried out, conclusions drawn from the experiments cannot be more than possible explanations.

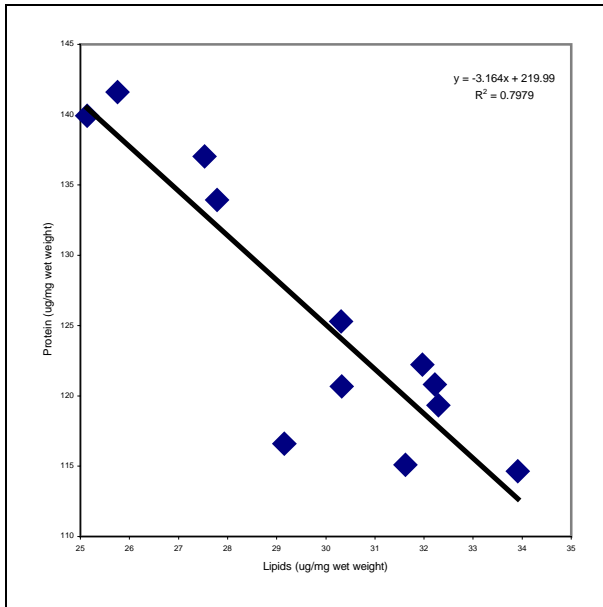


Figure 16: Relationship between lipid contents and protein contents as measured in the experiment. Note that the outcome of the experiment suggests a linear relation between lipids and protein. This relation would be typical for a conformation response.

6 RNA/DNA ratio as an instrument of growth measurement for juvenile signal crayfish, *Pacifastacus leniusculus*

6.1 Introduction

Crayfish, like other crustaceans can only grow by periodical moulting. As members of the group of *Arthropoda* crayfish follow a diversified basic arthropod body plan. In common with all other arthropods, crayfish can increase in length only by periodically shedding the exoskeleton during the process of moulting. The life cycle of crayfish progresses through a series of moults interspersed by intermoult. Crayfish increase the water content of their body during moulting and the amount of water in the tissue can lead to biased growth data when not taken into account.

The replenishment of water with newly built body tissue that takes place during intermoult depends on the amount and quality of nutrients available at the time.

As the crayfish get older the number of moults per growth season decreases. However, the nutritional status of the animal and the conditions of temperature and light under which it is kept influence the process (Aiken, 1969; Lowery, 1988).

Due to moulting the determination of age and growth of crayfish is difficult since the animals do not retain any permanent features such as the growth rings on the scales of fish.

Precise data and growth rates are difficult to obtain from the field because it is almost impossible to determine the age of an animal picked up at random. This is especially true in the case of juvenile crayfish.

In order to assess the effect of environmental factors on wild populations it is therefore necessary to combine both field data and experimental laboratory studies.

Many reports have been published showing the influence of different factors on crayfish growth and survival (Savolainen *et al.*, 2004; Sáez-Royuela *et al.*, 1996; Tulonen *et al.*, 1995).

However, most studies had to rely on indirect methods for growth measurement and as stated, measurements did not take into account short-term changes in the animals' condition as a reaction to a changing environment (Marsden *et al.* 1973; Jussila and Mannonen, 1997).

Measurements of crayfish growth in terms of weight and length are therefore always influenced by the moulting cycle of the animals.

In aquaculture, it is mainly the type and quality of the diets available to the animals and the bio availability of nutrients at the right time that influence the growth and survival of the animals. Stocking density and outer factors as temperature and light are often controlled to match the needs of the crayfish but dietary research has been neglected in the last decades. Especially for crayfish and other crustaceans, new approaches are called for taking into account the special growth patterns of these animals. New methods complementary to weight and length measurements are needed and there are several choices like direct protein synthesis, respiration, but many of these pose constraints to the type of experiments they suit. RNA/DNA on the other hand is quite flexible and has been previously used for other animals (Clemmesen, 1993; Vrede *et al.*, 2002), and is therefore very potential candidate for crayfish research.

The measurement of RNA has already been used earlier to determine the nutritional status of crayfish (Edsman *et al.*, 1994) and other crustaceans (Rosa and Nunes, 2003). These studies focussed on RNA content as a measurement of metabolic activity as it may also be used as a reference for protein synthesis.

While the amount of DNA in a cell remains unchanged during the process of protein synthesis, the amount of RNA present is highly dependent on the amount of synthesis taking place at that particular moment. In contrast to the measurement of RNA content carried out by Edsman *et al.* (1994), RNA/DNA ratio measurements provide an additional baseline by including the constant level of DNA present in the animals' tissue.

Research has been carried out presenting a variety of suitable methods for the determination of protein synthesis and degradation (El Haj *et al.*, 1996; Fraser *et al.*, 2002) and methods like the utilisation of radioactive isotopes is widely used in protein synthesis research.

However, these usually require constraint environments for measurements. The applicability of a highly sophisticated method like protein synthesis measurement is not given for research carried out in all research facilities. Sampling and measurements have to be kept simple to increase the acceptance of the method in field research.

The RNA/DNA ratio measurement may be more flexible and suitable for various types of growth experiments.

Introducing a method like RNA/DNA ratio measurement to crayfish research would open opportunities to a far more detailed understanding of crayfish growth and nutrition. A direct measurement of the animals' response to different sources of nutrients as well as possible fluctuations of synthesis rates during intermoult can be examined in a more detailed way with the RNA/DNA ratio measurement.

The reliability of the RNA/DNA ratio measurement makes it popular also in other fields of research and several methods have been developed and published (Gorokhova and Kyle, 2002; Shin *et al.*, 2003; Vrede *et al.*, 2002; Kyle *et al.*, 2003). Little work has been carried out with crustaceans and the method has not been tested with crayfish so far. It was therefore an additional challenge to introduce the method of RNA/DNA ratio measurement into crayfish research and provide new possibilities in growth assessment for crayfish.

The method used in this study is based on the fluorospectrometric measurement of RNA and DNA coloured with ethidium bromide. The method for fish and fish larvae survey was described by Clemmesen 1993 and has been widely used with fish so far. Detailed information about the method is given in *Chapter 3 “Material and methods”*.

6.2 Experimental set-up

The experiment was carried out using 16 tanks of the experimental system. Tanks were randomly chosen and stocked with 20 animals each, resulting in a stocking density of 40 animals/m². Before the animals were weighed and measured to start the experiment, they were given two weeks for acclimatisation. The experiment was carried out in a controlled environment over a period of three months. Water temperature was kept within a range from 17.5 to 19.0°C and light conditions were fixed to 18L:6D photoperiod. All tanks were cleaned on regular basis and uneaten food, exuviae and other remains were removed from the tanks daily (except on weekends).

When starting the experiment, tanks were divided into four treatment groups at random. These groups were to imitate different food availabilities and thus reinforce different metabolic responses. Specifically, the number of feedings per week was varied. The different treatment groups were fed according to a strict regime to monitor short-term changes in the animals' RNA/DNA ratio caused by different stages of starvation.

The detailed feeding plan for the different treatment groups is given in *Table 10*.

Feedings	Weekdays				
	per week	Monday	Tuesday	Wednesday	Thursday
5	Feed	Feed	Feed	Feed	Feed
3	Feed	xxx	Feed	xxx	Feed
2	Feed	xxx	xxx	xxx	Feed
1	xxx	xxx	Feed	xxx	xxx

Table 10: RNA/DNA ratio as an instrument of growth measurement. Feeding schedule for the experimental groups of this experiment. The “Feed” entries represent the feeding days for the specific treatment group and the “XXX” entries stand for days without feeding. The groups are described by the number of weekly feedings; no feeding took place on weekends. Every treatment group consisted of four tanks, stocked with 20 animals each.

All animals received the same diet. The diet used in this experiment was identical to the ones used in most of the experiments of this study. Raw ingredients were fishmeal (54.0%), fish oil (2.5%) and wheat-starch (31.0%). Additionally, a base mix (12.5%) containing vitamins, minerals and carotenoids, was added to the ingredients. The base mix also contained Finnfix as a binder (2.0%). Raw ingredients were mixed with 350ml of purified water and the dough was processed with a Kenwood household processing machine. Pellets were air-dried at 30°C for 48h in an oven before being bagged and kept in the refrigerator until use.

The diet composition was calculated using the nutritional values of the raw ingredients as described by Ruohonen and Vielma (1994). Details are given in *Chapter 3 “Material and methods”*, *Table 2*. The formulation resulted in 38.8% protein, 7.6% fat, 27.8% carbohydrates and fibre and 6.0% ash. The water content was 7.3% and all values are given on as-fed basis. Animals were fed approximately 5% (diet on as-fed basis) of their initial body weight with each feeding, i.e. the amounts of diet given to the animals were not changed during the three months experimental period.

The experiment was terminated by measuring animals’ final wet weight and recording their carapace length. Growth data were calculated from these results on the basis of tank means (N = 4).

Animals were then killed and stored at –35°C for further measurements.

Subsamples of 5 animals from each tank were taken for the determination of RNA/DNA ratio. The RNA/DNA ratio was measured using muscle tissue from the abdominal muscles of individual crayfish.

Removing the exoskeleton from the frozen animals allowed taking tissue samples of 250mg from the abdominal muscle. These samples were then used for the determination of RNA and DNA contents, which led to the calculation of the RNA/DNA ratio. Details about the measurement are given in *Chapter 3 “Material and methods”*. RNA/DNA ratio data are therefore based on 5 animals per tank, resulting in 20 animals per treatment group (N= 20) (for animals fed only once a week, treatment 1, N = 15).

Statistical analysis that included the use of general linear models were carried out for the measurements of growth in weight and carapace length increment as well as for RNA/DNA ratio using the “R” language (The R Foundation of Statistical Computing, 2003). The various feeding regimes were used as treatments.

6.3 Results

During the experimental period, one of the tanks chosen for this experiment showed some abnormally high mortality and animals in this tank were not growing and moulting. When the mortality increased 25% of all animals in the tank, it was decided to exclude the specific tank from the experimental results. Therefore, all feeding groups consist of four replicates with the exception of this group of animals fed once a week. Due to the loss of one tank, only three replicates of this treatment could be taken into the evaluation of the experimental outcome.

Initial carapace lengths varied from 24.92 to 28.11mm and initial wet weight from 4.34 to 6.16g.

Final wet weights ranged from 6.78 to 9.53g and carapace lengths from 28.77 to 32.00mm.

Detailed information about the results of the experiment is given in *Table 11*.

Measurements	Weekly feedings			
	1	2	3	5
Initial carapace length (mm)	26.72	26.07	26.80	26.93
SD	1.58	0.85	0.93	0.88
Skewness	-1.56	0.97	1.33	-1.64
Kurtosis	NA	1.35	1.71	2.89
Initial wet weight (g)	5.31	4.90	5.36	5.47
SD	0.73	0.52	0.58	0.64
Skewness	-0.97	1.02	1.18	-1.22
Kurtosis	NA	1.33	0.55	1.36
Final carapace length (mm)	30.04	30.04	30.61	31.32
SD	2.03	0.28	0.59	0.71
Skewness	-1.58	0.40	1.19	-1.21
Kurtosis	NA	-3.49	2.22	2.02
Final wet weight (g)	7.78	7.73	8.69	9.10
SD	1.03	0.19	0.51	0.73
Skewness	-0.65	-0.17	-0.86	-1.98
Kurtosis	NA	-1.43	-1.28	3.92
Carapace length increment (mm)	3.32	3.97	3.82	4.39
SD	0.45	0.76	0.62	0.51
Skewness	1.66	-0.92	1.76	-0.17
Kurtosis	NA	1.83	3.07	1.40
Wet weight gain (g)	2.75	2.83	3.33	3.63
SD	0.31	0.45	0.59	0.24
Skewness	-1.65	0.57	0.36	-0.60
Kurtosis	NA	0.47	-3.85	-1.40
RNA/DNA	6.64	7.19	8.35	8.89
SD	0.23	0.23	0.25	0.25
Skewness	0.19	0.62	1.89	1.30
Kurtosis	NA	1.61	3.63	1.23

Table 11: RNA/DNA ratio as an instrument of growth measurement. Mean values standard deviations, skewness and kurtosis for the measurements carried out in this experiment. The values represent the means of the different treatment groups. The number of weekly feedings describes the groups; no feeding took place on weekends. Every treatment group consisted of four tanks ($N = 4$), stocked with 20 animals each, the group of animals fed once a week consisted of 3 tanks ($N = 3$).

Small differences in final carapace length that were detected at the end of the experiment between the feeding regimes were not statistically significant ($p = 0.6867$, $F = 0.1702$). The residual standard error was 0.59 with 13 degrees of freedom.

Carapace length increase varied from 3.32 to 4.39mm. Animals fed once a week showed slowest growth, animals fed twice or three times a week grew quite similarly with 3.97 and 3.82mm carapace length increment.

As expected, the animals fed five times a week gave the best average result in terms of carapace length increase (4.39mm).

Increments in wet weight ranged from a minimum of 2.32 to a maximum of 4.20g with average values of 2.75g for the animals fed once in a week and 3.63g for animals fed five times a week. The animals fed twice or three times a week respectively had an average wet weight gain of 2.83 and 3.33g.

Statistical analysis showed significant effects of feedings on the wet weight gain during the experiment ($p = 0.01012$, $F = 9.0355$). The residual standard error was 0.41 with 13 degrees of freedom.

The RNA/DNA ratios varied from 6.71 for animals fed once, 7.19 for animals fed twice to 8.35 for animals fed three times in a week. The highest RNA/DNA ratio was measured for the animals fed five times a week (8.89) (*Table 11*). The statistical analysis revealed a significant influence of the feeding regime on the outcome of the measurement ($p = 8.128e-08$ and $F = 102.27$). The residual standard error was 0.34 with 14 degrees of freedom.

A graphical overview of the results of the experiment is given in *Figures 17, 18 and 19.*

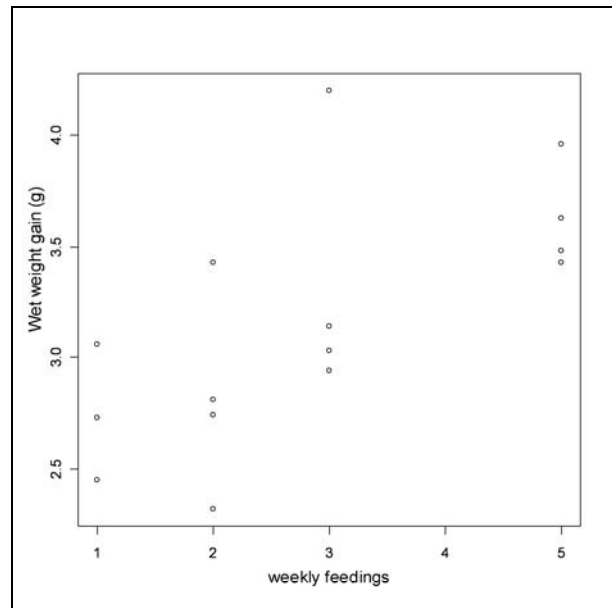
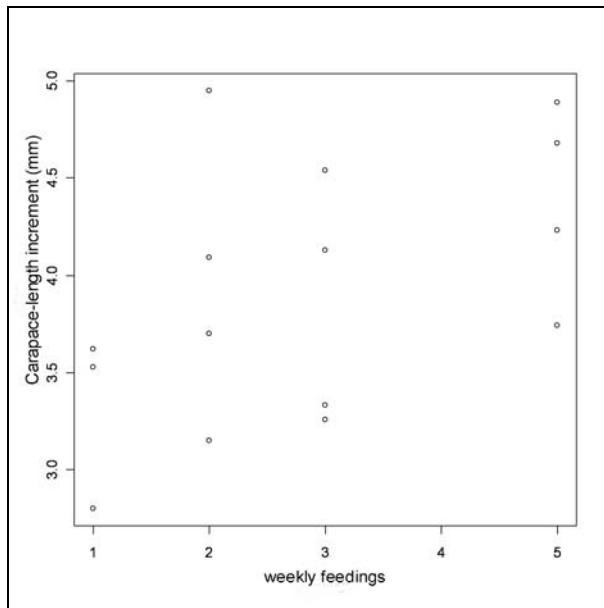


Figure 17 (left): RNA/DNA ratio as an instrument of growth measurement. Influences of feedings on carapace length increment (mm) measured at the end of the experiment. The values represent the means of each tank in the different treatment groups. The number of weekly feedings describes the groups; no feeding took place on weekends. Every treatment group consisted of four tanks ($N = 4$); treatment 1 consisted of three tanks.

Figure 18 (right): RNA/DNA ratio as an instrument of growth measurement. Influences of feedings on wet weight gain (g) measured at the end of the experiment. The values represent the mean values calculated for the different treatment groups. The number of weekly feedings describes the groups; no feeding took place on weekends. Every treatment group consisted of four tanks, $N = 4$. Treatment 1 consisted of 3 tanks only.

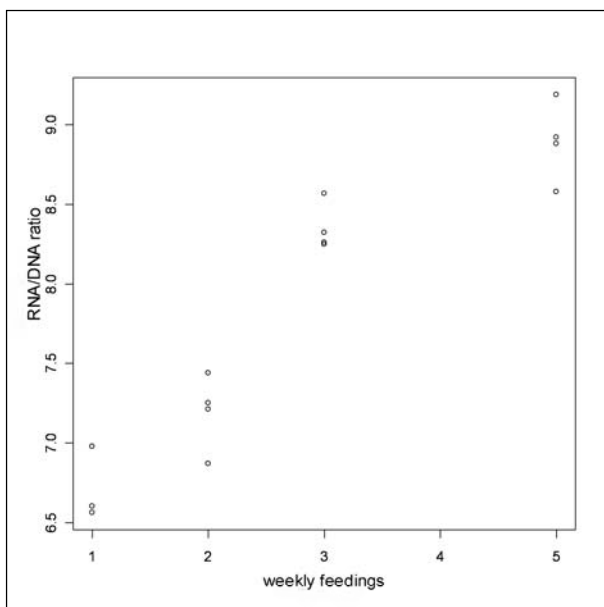


Figure 19: RNA/DNA ratio as an instrument of growth measurement. Influences of feedings on final RNA/DNA ratios measured at the end of the experiment. The values represent the mean values calculated for each tank of the different treatment. The number of weekly feedings describes the groups; no feeding took place on weekends. Every treatment group consisted of four tanks. $N = 20$, for treatment 1, $N = 15$

6.4 Discussion

In common with all other arthropods, crayfish can increase in length only by periodically shedding the exoskeleton during the process of moulting or ecdysis (Holdich and Reeve, 1988; Guan and Wiles, 1999; Gydemo and Westin, 1993). The life cycle of crayfish progress through a series of moults interspersed by intermoult. Due to moulting the determination of age and growth of crayfish is difficult since the animals do not retain any permanent features such as the growth rings on the scales of fish (Lowery, 1988).

Weight gain takes place during the moult itself, when crayfish takes water into the tissues and thus increases in size, weight and volume. The weight gain at moult is normally between 30 and 60% of premoult weight. During intermoult the crayfish substitute their water content with tissue growth, and the weight gain during the long intermoult is normally less than 5% of immediate postmoult weight (Jussila, 1997; Huner and Barr, 1991; Huner, 1995).

The experiment dealing with the effects of feeding regime on RNA/DNA ratio clearly followed the anticipated short-term changes of metabolic rates in crayfish fed differently. Feeding regime has proven to play a major role in crayfish aquaculture and the outcome of this experiment has stressed the importance of well planned feeding. Even though, Meade and Watts (1996) suggest that short-term nutrient deprivation may not have long-term consequences resulting in stunted populations, an influence of nutrient deprivation on RNA/DNA ratio was clearly measurable. Crayfish fed on daily basis showed significantly higher RNA/DNA ratios (8.89) representing higher rates of protein synthesis than animals fed less often. Similar results were obtained by Vrede *et al.* (2002) and Gorokhova and Kyle (2002) both working with *Daphnia*.

Edsman *et al.* (1994) has described changes in the animals' RNA content similar to the results measured in this study. A comparison of the RNA concentrations measured revealed high similarities between the results of Edsman *et al.* and the present work. Edsman *et al.* found RNA levels of 0.86mg/g ww for crayfish fed continuously and 0.69mg/g ww for animals starving for at least 2 days while the RNA levels measured from this experiment varied from 0.58mg/g ww to 1.00mg/g ww depending on the feeding frequency.

Metabolic rates decreased already significantly after one day of starvation and reach a very low level when the animals are fed only once or twice a week. All groups had a two days starvation period during the weekends, which has certainly had an additional influence on growth rates and metabolic rates. Even though this development sounds logical, differences in wet weight and especially carapace length are far less obvious than the short-term changes measured for

RNA/DNA ratio. They followed a similar trend but for carapace length the pattern was not even statistically significant. It appears that assessment of crayfish growth in terms of weight and length only may possess low statistical power for experiments. This could explain why several studies, e.g. in this study, show statistically insignificant results.

Results gained from the measurement of weight and carapace length show small differences between animals fed only once and animals fed twice a week. These results suggest the necessity of long-term studies to monitor the influence of feeding regime on the animals' development. Mid-term experiments lasting up to three months seem still insufficient to gain representative data even though the experiments are carried out using juvenile crayfish. Due to their biology, crayfish growth is always dependent on moultings. The results of this study support the hypothesis that real replenishment of tissue requires a high number of moults, thus a mid-term experiment cannot represent the physiological responses to different factors adequately (Mannonen, *pers com.*, Huner, *pers. com.*).

Animals not fed on regular basis will need significantly more energy for their survival than animals fed five days a week. The rates of protein synthesis can only be kept at a high level, when the flux of nutrients required is maintained (*Fig. 20*). RNA/DNA ratios measured suggest that daily feeding should take place. These results are underlined by the growth rates measured for the experiment. For wet weight increase and carapace length increment daily feeding showed best growth results. Animals fed three times a week resulted in an average gain in wet weight of 3.33g and a growth in carapace length of 3.82mm and are clearly outperformed by the animals fed on daily basis. These were measured with a mean wet weight gain of 3.63g and an average carapace length increase of 4.39mm.

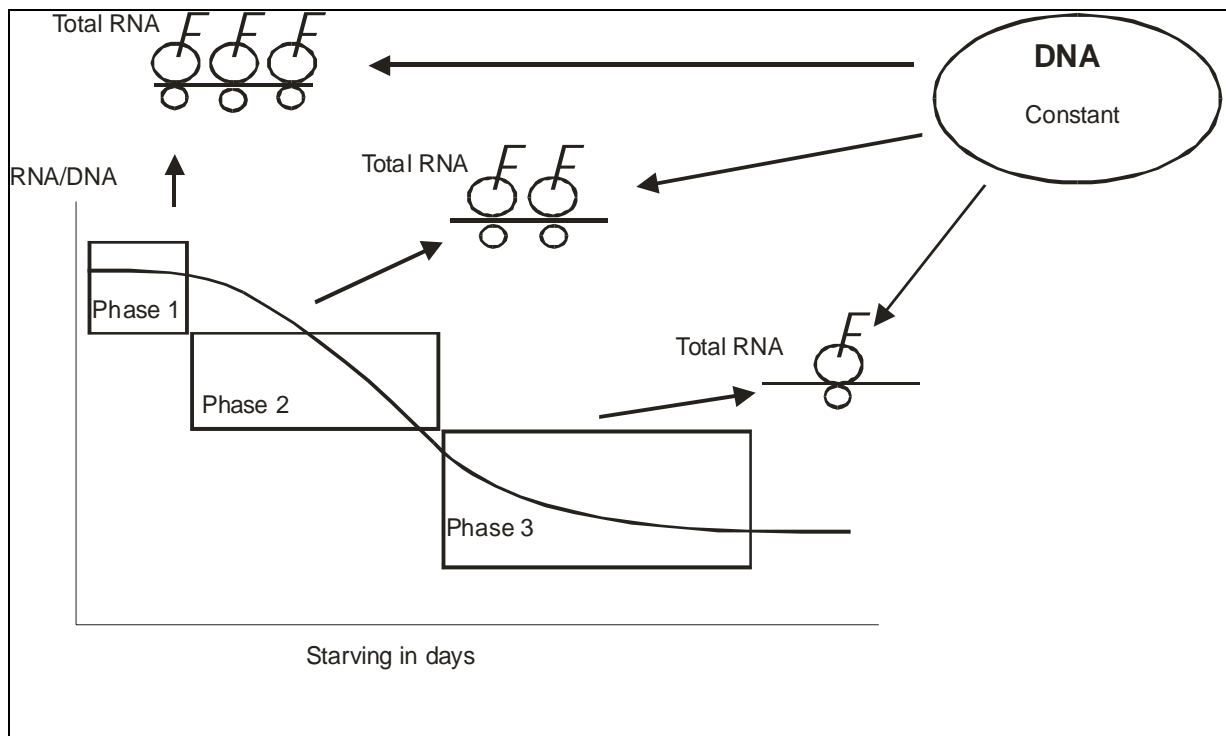


Figure 20: RNA/DNA ratio as an instrument of growth measurement. Schematic presentation of the influences of starvation on RNA/DNA ratio. After a phase where high RNA/DNA ratio can be measured, starvation leads into a decline of RNA/DNA ratio (Phase 2) and finally reaches a bottom line where no further decline will be possible (Phase 3) (Clemmesen, modified)

The experiment clearly showed how the differences in RNA/DNA ratio related to feeding regime and availability of nutrients could be used to measure the physiological responses of freshwater crayfish to changes in their environment. The measurements have proven also the relationship between RNA/DNA ratio measured and body-mass produced.

Further measurements to improve the reliability of the method need to be carried out and other aspects influencing the RNA/DNA ratio have to be kept in mind. It is suggested that crayfish other aspects influencing the RNA/DNA ratio have to be kept in mind. It is suggested that crayfish should not be measured for RNA/DNA ratio in phases of pre- and postmoult because the reliability of the measurements might be greatly influenced by the animals' physiological condition. Therefore, the most stable intermoult period seems to be most suitable for sampling. The measurements carried out for this experiment did not include any soft-shelled animals that were preparing to moult or had just successfully moulted. However, the study of changes in RNA/DNA ratio during the entire moulting cycle will certainly be another great challenge for future research. RNA/DNA ratio measurement may soon be established as a standard measurement in freshwater crayfish research. Many different environmental factors like pH, oxygen saturation, temperature and light intensity can influence the metabolism of crayfish (Chittleborough 1975, Aiken 1980, Hartnoll 1983, Botsford 1985, Lowery, 1988). It will be an

interesting challenge to examine the influence of several environmental factors on the RNA/DNA ratio. A further attempt to prove the feasibility of the method was its use to deliver additional information about the effects of different binding agents on stability of pelletised diets, on their digestibility and thus crayfish growth.

7 Effects of diets using different binding agents on growth of juvenile signal crayfish *Pacifastacus leniusculus* and their RNA/DNA ratio

7.1 Introduction

Crayfish like most aquatic crustaceans tend to feed relatively slowly and intermittently. Different kinds of diet manipulation like handling with the chelae, tearing the diets apart etc. have been monitored before ingestion (D'Abramo *et al.*, 1997; Jussila, *pers. com*; Mannonen, *pers. com*). The variability in feeding behaviour and slow feeding rate make it necessary to prepare diets that meet the animals' requirements not only in terms of nutritional value but also in terms of availability and digestibility (Ilheu and Bernardo, 1993; Mitchell *et al.*, 1995; Ruohonen and Wolf, 2000; Söderbäck *et al.*, 1987; Warner *et al.*, 1995). Therefore, diets prepared for these animals should be bound firmly enough to remain intact in water for a prolonged period and to resist disintegration during feeding manipulations (D'Abramo *et al.*, 1997) without compromising the bioavailability of nutrient or the chemical stimulus for feeding behaviour. Research on various binding agents has been undertaken on both fish and crustacea. Some binding agents have been evaluated earlier (Storebakken and Austreng, 1987; Ackefors *et al.*, 1992) and Heinen (1981) published a study dealing with the effects of different binding agents on stability of possible crustacean diets. In his study, Heinen (1981) compared 11 different binding agents that were used in dry weight concentrations of 3% or less. Diets prepared from those binders reached stabilities from less than 6 hours (Cornstarch) up to 24 hours and more (Keltone). Although ruling out some of the binding agents' suitability for crayfish diets, Heinen's results focused only on the stability of the diets and did not take into consideration the effects of different binders on digestibility and, thus, bioavailability of the nutrients or ultimately on growth.

7.2 Preliminary measurements

This experiment was designed to focus on the effects of different binding agents on growth and RNA/DNA ratio of juvenile crayfish. Preliminary experiments were undertaken to ensure that selected binding agents were suitable for the preparation of a dry pelletised crayfish diet and that binders could be used in small amounts to ensure the nutritional value of the diets was not

harmed. High concentrations of binding agents result in a lack of nutritional value due to the replacement of nutrient by binding agents and more likely by reducing the digestibility of nutrients. Even though binders are not nutritionally inert, their nutritional value can be neglected for this experiment due to low concentrations used.

Preliminary experiments were carried out in the laboratory to monitor the stability of different binders in water. For this purpose, a small variety of potential binding agents were chosen and diets were prepared as described below. The binding agents used in this experiment were partly chosen on the basis of the paper by Heinen (1981) and also some new agents were used for comparison.

The binders used are presented in *Table 12*.

Binding agent	Description	Manufacturer
Agar agar	Polysaccharide	KEBOLab, Sweden
Finnfix 50000	Carboxymethylcellulose	Serla, Finland
Hygum	Iota type carrageenan	Hydralco Ltd, Germany
Keltone	Refined sodium alginate, Requires a sequestrant	ISP Alginates Ltd, U.K.
Lignobond FPM	Sodium Lignosulfate, Derived from spruce wood sulphite liquor	Borregard Lignotech, Finland

Table 12: Effects of different binding agents. Detailed information of the different binding agents used in the preliminary tests. Notice that Lignobond was not used in the actual trial due to its unsatisfactory binding abilities.

A cold dough was used for all diets except the one using agar agar as a binder for which hot water was used to reach the binding effect. Keltone was used in a concentration of 2.50% dry weight complemented with 0.50 % of sodium hexametaphosphate as a sequestrant necessary for this binder. Alginates are hydrophilic colloids and are widely used in the food industry. In a great number of food applications, the now well-known reactivity of alginates with calcium ions is utilised.

The sequestrant is used for the inhibition or prevention of metallic ion precipitation from the mixture by formation of a coordination compound with its phosphate. In most situations, calcium release during the mixing of the ingredients is so rapid that a calcium sequestrant is required to control the reaction by competing with the alginate for calcium ions. Typical food-approved sequestrants are sodium hexametaphosphate, tetrasodium pyrophosphate, and sodium citrate (ISP, 2004).

The diets contained wheat-starch (31.0%), fishmeal (51.5%) and fish oil (2.5%) as well as a base mix (12.0%) containing vitamins, minerals and carotenoids. Binding agents were added in a concentration of 3.0% of dry weight and the pastry was processed with a household food processor (Kenwood Ltd). Blocks of diet were formed and were then dried in an oven at 30°C for 48 hours.

The dried blocks were weighed and cut to a constant weight of 3.5g. To measure their binding properties, the sample blocks were then placed into beakers containing 200ml of purified water, and mechanical manipulation was simulated using a magnetic stirrer at low speed (<100 rpm). Samples were continuously stirred at room temperature (21°C) for 12 hours and the disintegration process was monitored after 1, 2, 4, 6 and 12 hours. Each diet was measured in 10 replicates. Disintegrated pellets were removed from the beakers and the remains were dried on petri dishes at room temperature for 24h.

The result of this preliminary experiment suggested the suitability of four binders for the preparation of a stable crayfish diet, although stabilities resulting from the experiment were significantly different (*Figure 21*). The diet prepared with Lignobond did not show any binding effect at all and started disintegration after several minutes. Therefore, the Lignobond-based diet was no longer tested and the sample was taken out of the results.

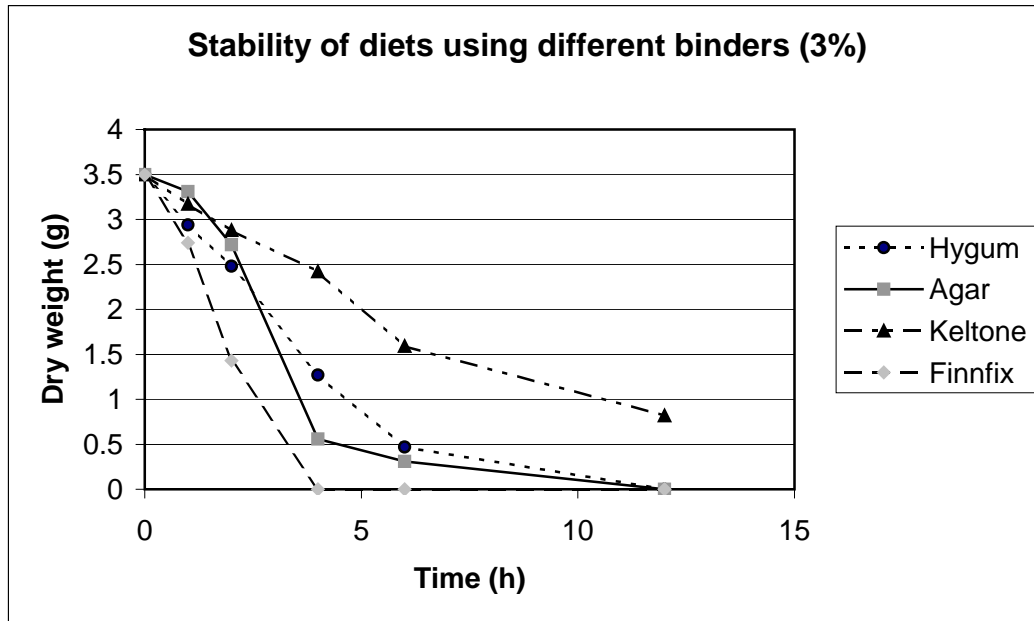


Figure 21: Results of the stability test using different binders in dry weight concentrations of 3%. Dry weight of disintegrated diets was taken after the samples had been removed from the experiment. Values in g represent the dry weight of the largest remains as mean values. N=10 for all binding agents.

At the end of the preliminary experiments, the four remaining binders were taken for the preparation of four batches of experimental food for a growth trial. Hygum, Agar and Finifix were used in dry weight concentrations of 3%. Keltone was used in a concentration of 2.5% with 0.5% sodium hexametaphosphate added as a sequestrant summing up to 3% of dry weight. The diets were prepared as described above and pelletised with a Kenwood kitchen food processor. The pellets were left to dry at 30°C in a drying oven (Termacks Ltd).

7.3 Experimental set-up

The experiment was set up in the experimental system with 16 tanks, each stocked with 20 juvenile crayfish. To ensure, environmental effects did not vary between the tanks, four replicate tanks were randomly allocated to each binding agent used. As mentioned above, all other ingredients of the diets remained the same. Again, the diets were produced from wheat starch (31.0%), fishmeal (51.5%) and fish oil (2.5%) as well as a base mix (12.0%). The formulation resulted in a calculated macronutrient composition of 37.1% protein, 7.3% fat, 27.8% carbohydrates and fibre and 5.7% ash. The water content was 7.1%.

Feeding took place on daily basis five times a week and animals were fed 5% of the initial body weight every day. Frequent cleaning of the tanks ensured that no unconsumed food remained in the tanks and fouling did not affect the water quality in the system.

Before the experiment was started, the animals were given 2 weeks for acclimatisation and 20 animals were then randomly chosen for stocking of each tank. At the beginning of the experimental period, all animals were measured for carapace length and initial weight.

The experiment was carried out over a period of 3 months to allow several moultings and thus the effects of the different binding agents to be measurable.

Dead animals as well as exuvia and other remains of food or animals were removed from the tanks daily and the controlled environment of the experimental system was used to create optimal temperature and light conditions for the animals. Water temperature was kept within 17.5 to 19°C and an 18L:6D photoperiod was chosen to ensure light conditions were suitable for the animals.

At the end of the experiment, all animals were measured for body weight and carapace length. The data gained were then used to evaluate the influences of the different binding agents on the animals' development. Weight and carapace length measurements contributed the data for the calculation of growth. Wet weight gain and carapace length increment were used for the comparison between the different treatment groups (N = 4 per group).

In addition, all animals were killed by deep-freezing and 5 animals of each tank were randomly chosen as subsamples that were taken to measure the RNA/DNA ratio (N= 20).

The method of RNA/DNA ratio measurement was used as a more sensitive method to detect if the different binding agents had any influence on the diets' digestibility and the bioavailability of essential nutrients consumed with the diet. Additionally, binders can influence feed intake affecting RNA/DNA ratios. (D'Abramo *et al.*, 1997) Unfortunately, methods measuring digestibility and feed intake are poorly developed in crayfish research and the animals' selective feeding behaviour has proven to cause major difficulties in measuring feed intake (Ahvenharju, *pers. com., unpublished data*).

The RNA/DNA ratio was measured using muscle tissue from the abdominal muscles of individual crayfish. The preparation of the samples took place using a cold store with 5°C room temperature.

Removing the exoskeleton from the frozen animals allowed taking tissue samples of 250mg from the abdominal muscle. These samples were then used for the determination of RNA and

DNA contents, which led to the calculation of the RNA/DNA ratio. Details about the measurement are given in *Chapter 3 “Material and methods”*.

Statistical analyses of growth data and the results of the RNA/DNA ratio measurements used general linear models. Analyses were and carried out using the “R” language (The R Foundation of Statistical Computing, 2003).

7.4 Results

Initial carapace lengths ranged from 22.33 to 23.56mm, wet weight varied from 2.83 to 3.52g. Final carapace length varied from 26.62 to 29.16mm. The animals fed a diet bound with Finifix showed the best result in terms of carapace length (28.40mm), followed by Agar (28.24mm), Keltone (27.87mm) and Hygum (27.82mm).

Final wet weights ranged from 5.20 to 6.97g with mean values of 6.40g for the animals fed a Finifix diet and 5.90g for animals fed the diet bound with Keltone. The animals fed Agar or Hygum diets respectively had an average wet weight of 6.16 and 6.11g.

The results of the experiment are given in *Table 13*.

Measurements	Binding agents			
	Agar	Keltone	Hygum	Finnfix
Initial carapace length (mm)	22.93	22.79	22.69	22.76
SD	0.54	0.17	0.39	0.23
Skewness	1.12	1.97	1.31	-0.33
Kurtosis	0.09	3.91	2.04	0.76
Initial wet weight (g)	3.16	3.22	3.15	3.20
SD	0.17	0.12	0.27	0.12
Skewness	-1.10	0.00	1.40	1.62
Kurtosis	2.06	-6.00	2.41	2.43
Final carapace length (mm)	28.24	27.87	27.82	28.40
SD	0.64	0.30	0.83	0.71
Skewness	-0.27	1.33	-1.65	-0.72
Kurtosis	-4.35	1.74	2.78	1.25
Final wet weight (g)	6.16	5.90	6.11	6.40
SD	0.33	0.36	0.63	0.52
Skewness	-0.68	1.91	-1.73	-0.73
Kurtosis	-1.38	3.69	2.93	1.72
Carapace length increment (mm)	5.31	5.08	5.13	5.64
SD	0.74	0.43	0.77	0.63
Skewness	0.72	0.01	-1.06	-1.67
Kurtosis	1.19	0.83	0.06	3.07
Wet weight gain (g)	3.00	2.68	2.96	3.20
SD	0.43	0.44	0.57	0.47
Skewness	0.51	1.66	-1.47	-1.60
Kurtosis	1.05	2.81	1.89	2.95
RNA/DNA	7.69	8.90	8.33	9.34
SD	0.49	1.26	1.99	0.99
Skewness	-1.73	-1.85	-0.67	-1.85
Kurtosis	2.98	3.43	-2.24	3.57

Table 13: Effects of different binding agents. Mean values standard deviations, skewness and kurtosis for the measurements carried out in this experiment. The values represent the means of different treatment groups. The different binding agents represent the treatment groups. N = 4

Calculated carapace length increments varied from 4.10 to 6.34mm.

Statistics proved that there was no significant effect of the binding agents on the growth measured in length (cl) in this experiment ($p = 0.64$, $F = 0.58$). Effect sizes were calculated with agar (5.35mm) as baseline: 0.28mm for the Finnfix group, -0.22mm for the Hygum group and –

0.27mm for the Keltone group. The residual standard error was 0.66 with 12 degrees of freedom.

Animals fed on the Keltone diet showed poorest length increment, followed by Hygum and agar. Best growth in length was obtained from the animals fed with the Finnfix diet.

Resulting growth in terms of wet weight gain of the animals varied from 2.15g to 3.60g.

Statistical evaluation did not reveal any significant effects of the different binding agents ($p = 0.49$, $F = 0.85$). The effect sizes were 0.15g for the Finnfix group, -0.08g for the Hygum group and -0.37g for the Keltone group with agar as baseline (3.05g). The residual standard error was 0.48 with 12 degrees of freedom.

Weight gain was highest for crayfish fed on Finnfix feed, followed by those fed with agar and the animals fed on Hygum feed. Animals fed on Keltone showed smallest weight gain.

A detailed graphical overview of the results of the experiment is given in *Figures 22, 23 and 24*:

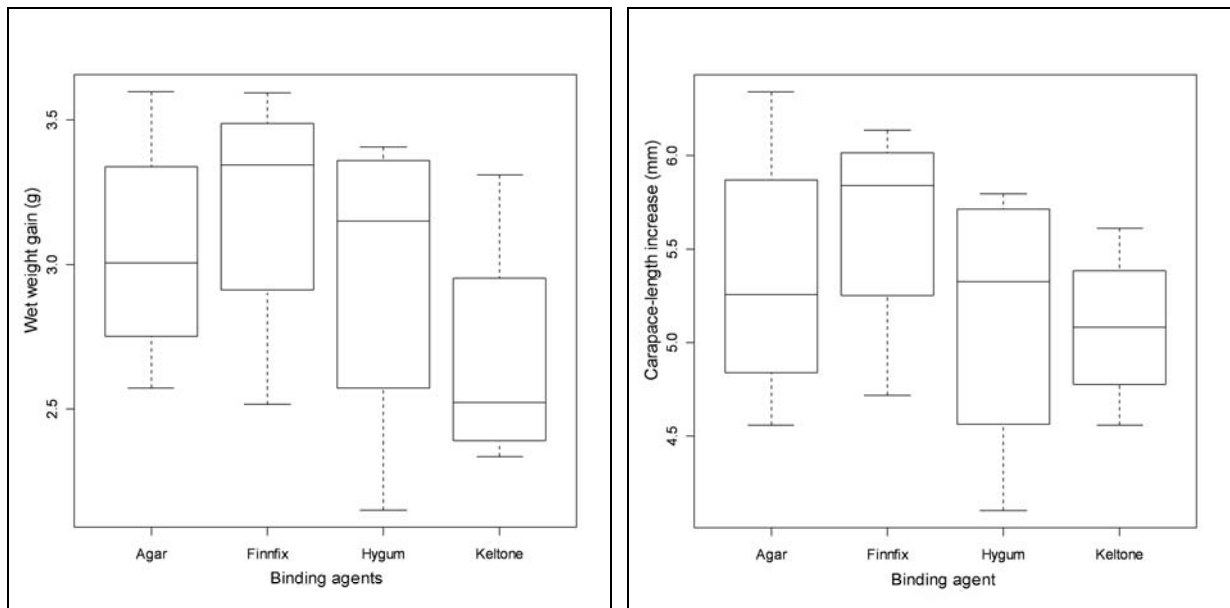


Figure 22 (left): Effects of different binding agents. Influences of binding agents on wet weight gain (g) measured at the end of the experiment. The error bars represent maximum and minimum values measured for the specific treatment group. The names of the binding agents describe the treatment groups. $N = 4$.

Figure 23 (right): Effects of different binding agents. Influences of binding agents on carapace length increase measured at the end of the experiment. The error bars represent maximum and minimum values measured for the specific treatment group. The names of the binding agents describe the treatment groups. ($N = 4$)

The measured RNA/DNA ratio reached from 6.66 to 10.03. The statistical evaluation of the RNA/DNA ratios did not reveal any significant differences ($p = 0.21$, $F = 1.78$). Effect sizes using agar (7.69) as baseline were 1.64 for Finnfix, 0.65 for Hygum and 1.22 for Keltone. The standard error calculated in the linear model analysis was 0.7547 and the residual standard error was 1.067 on 12 degrees of freedom. The difference in RNA/DNA ratio between the agar fed group and the Finnfix fed group was 21.3%, which is clearly biologically meaningful. It seems that although the number of replicates in this experiment was high, four, the statistical power of this wasn't sufficient.

The results of the RNA measurement are presented in *Figure 24*.

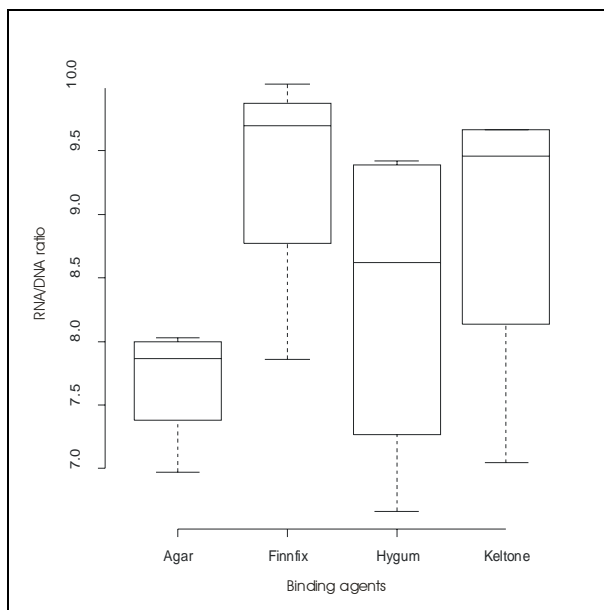


Figure 24: Effects of different binding agents. Influences of binding agents on final RNA/DNA ratios measured at the end of the experiment. The error bars represent maximum and minimum values measured for the specific treatment group. The names of the binding agents describe the treatment groups. RNA/DNA ratio was measured taking sub-samples ($N = 20$).

7.5 Discussion

The effects of different quality nutritional sources on RNA/DNA ratio were part of the experiment dealing with different binding agents. Although the nutritional values of all diets offered were the same, important, although not statistically significant, differences resulted from the different binding agents used in diet preparation. Not only did the different binding agents turn out to deliver huge differences in stability of the diet, but also an influence on

bioavailability of the nutrients could not be ruled out. Therefore, it was important, not only to measure the growth of the animals but to investigate the effects of the different binding agents on the animals' metabolism. Interestingly, it turned out, that the most stable diets had quite high outcomes in RNA/DNA rates as well. Agar however seems to reduce the digestibility of the diet or the availability was shortened resulting in lower RNA/DNA ratios (7.87). Hygum and Keltone formed the most stable diets and turned out to result in RNA/DNA ratios of 9.45 for Keltone and 8.62 for Hygum. The cellulose-based Finnfix however supported best RNA/DNA ratio (9.52), while being the weakest binding agent.

Taking into consideration the actual body growth obtained from the animals, the results have to be seen from a different angle. Even though the poor performance of agar can be seen from the growth data as well, the strong binding agent Keltone results in surprisingly low growth of the animals in the experiment. The Keltone bound diet returned poorest growth data, followed by Hygum and Agar. Finnfix resulted in highest wet weight gain (3.20g) and carapace length increment (5.64mm). This agrees with the measured high ratios of RNA/DNA.

The surprisingly weak performance of the alginate binders agar and Keltone seems to have several reasons. While Keltone-bound diets resulted in high RNA/DNA ratio and delivered weak growth figures, agar-bound diets deliver satisfactory growth while the RNA/DNA ratios stayed low. With agar, the digestibility of the diet might have been altered but nutrients could still be digested without influencing the anabolic/catabolic balance.

On the other hand, it can be suggested that the digestion of diets bound with an alginate binder like Keltone requires a high input of energy and the high RNA/DNA ratios measured do not result from protein synthesis used for tissue growth but from digestive metabolism. Even though measurements were taken from muscle samples, the small size of the animals might have led to a contamination of muscle tissue. The poor growth measured could then be seen as a result of high metabolic activity required for the digestion of the offered diets. Total costs of digestion might be almost as high as the energy in the meal. In this case, anabolism and catabolism would be out of balance, not allowing the animal to grow well even though a high rate of metabolism can be measured. Catabolism of nutrients releases energy (*Fig. 26*). This energy can be derived from different nutritional sources (*Fig. 25, left*) that are digested to supply the organism with energy and molecules needed for the "construction" of proteins, nucleic acids etc. The anabolism describes this energy-requiring process. The products of the catabolic digestion are used for processes like protein synthesis, tissue growth etc. that are described as anabolic. An influenced digestibility of the diet due to the alginate binders used in the

preparation could therefore lead to an increased anabolic activity prior to the catabolic digestion of the nutrients in the diet and thus decrease the nutritional value of the diet.

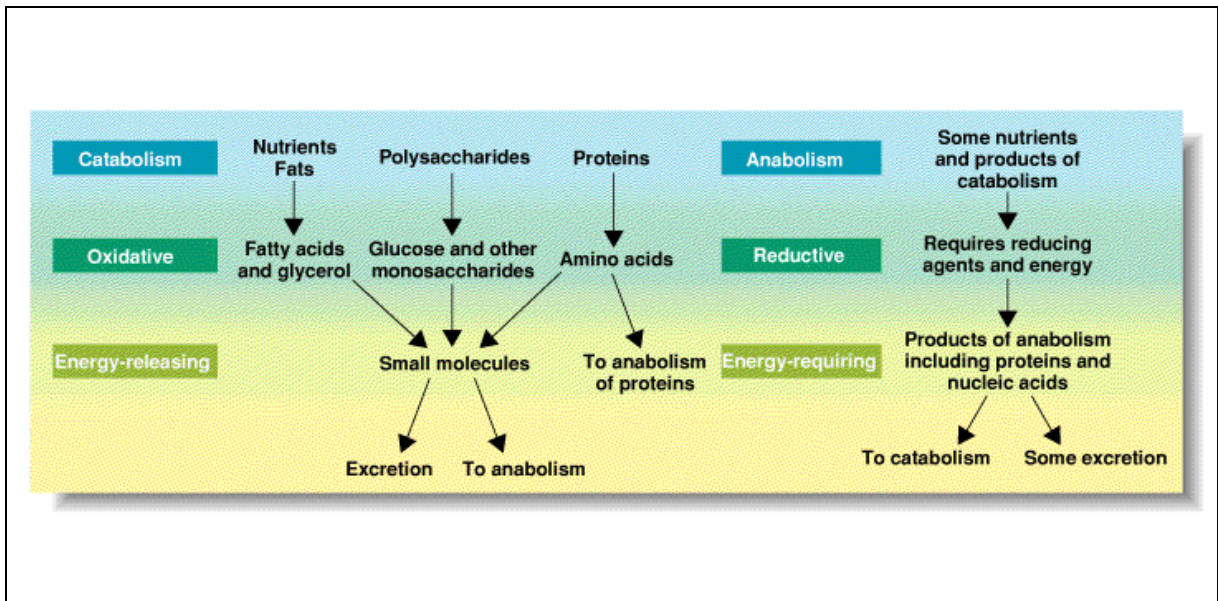


Figure 25: Catabolism and anabolism. An overview. (Campbell, 1998)

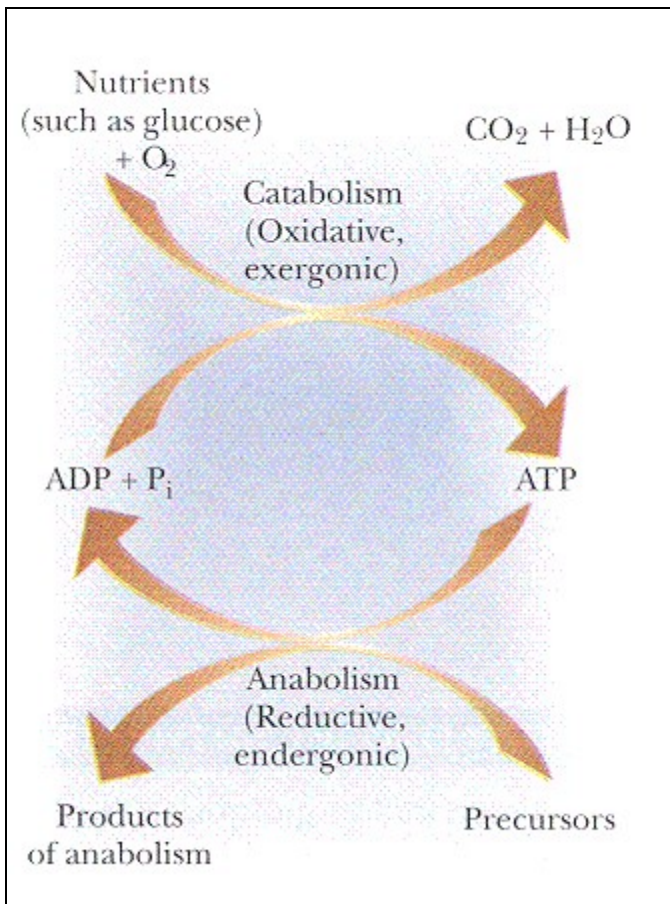


Figure 26: Catabolism and anabolism as processes producing and using energy. (Campbell, 1998)

Another explanation for the poor performance of Keltone as a binder could be given by the influence of alginates on the moulting cycle of crustaceans. The dependence of the moulting period on several factors has been described (Aiken, 1969; Lowery, 1988) and the results published explain the vulnerability of crayfish moulting periods to different factors. Studies on crustacean growth have shown that acclimation, dissolved oxygen concentration, food supply, isolation, temperature, photoperiod, sexual maturity and age could have a selective effect on weight increment at moult or length of the intermoult (Chittleborough, 1975; Aiken, 1980; Hartnoll 1983; Botsford, 1985). Stocking density or tank size have also shown to affect growth (Aiken 1980; Morrissy, 1992; Savolainen *et al.*, 2003).

An alteration of the intermoult period due to poor bioavailability of the nutrients bound with an alginate binder like Keltone could explain the poor growth results monitored for this binder as well as the high RNA/DNA ratio measured. Enzymatic activity is high in premoult, when water is taken into the tissue and the animals have to synthesise polysaccharide layers of their new exoskeleton. The nutrient profile affected both weight gain at molt and intermoult period in a study carried out by Jones *et al.* (1995) on yabbies (*Cherax destructor*). Furthermore, feeding ratio affected both intermoult period and weight gain at molt, though the influence on intermoult period was greater in the prawn (*P. elegans*) (Salama and Hartnoll 1992). D'Abramo *et al.* (1997) mention the effects of alginate binders on bioavailability and digestibility of protein and lipid. The results obtained from this experiment would support this hypothesis. Animals fed on Keltone bound diet had presumably not completed their moulting cycle prior to the measurements. At the time, when the animals were killed, they might have been in a stage of premoult, which would explain the high RNA/DNA ratio and the poor growth results. Unfortunately, no measurements of dry matter content were carried out in this experiment. Increased water content would have given additional support for this hypothesis.

The results support the observation of crayfish as slow feeders but the problem of digestibility and metabolic balance cannot be ignored when preparing diets meeting these requirements. Diets available over a long period of time can only result in good metabolic rates and thus in good growth if they do not affect the bioavailability of the nutrients and if their digestion does not result in an unfavourable catabolic/anabolic balance. The indifferent measurement of metabolic activity by the ethidium bromide method certainly can be seen as a weak point of the methodology. In addition to conventional measurements of growth it can help to explain the

reasons of different levels of performance. However, RNA/DNA ratio maybe high in connection to pre- and postmoult due to other reasons (El Haj *et al.*, 1996) than high growth activity and therefore measurements should focus on intermoult.

Even though the measured differences between the four binding agents Keltone, Hygum, Finifix and Agar in terms of wet weight gain and carapace length increase were small, it has to be stated that the differences were large enough to rule out the alginate binder Keltone as well as agar as suitable binding agents for crayfish diets. Keltone was unable to support good growth and the significant influence it has on the animals' metabolism shows a lack of inertness by influencing the digestibility to a serious degree. Agar unfortunately provides other problems. Agar-bound diets started fouling first in the experiment and their handling is too complicated. Especially the high hydroscopic level of Agar-bound diets is a severe problem in aquaculture and the observed fouling makes storage over a longer period very difficult.

The effects of binding agents on diet stability were outweighed by the influences of the binders on digestibility and nutrient availability. Even though suggestions are made that crustacean feeds should be as stable as possible (D'Abramo *et al.*, 1997), the interactions of binding agents with the nutrients in the diets have been studied and alginate binders are known to affect protein and lipid digestibility in fish (Storebakken and Austreng, 1987). The importance of feed stability occurs to be overestimated when dealing with crayfish in small experimental systems. Crustaceans use chemoreception to find their food sources. Even though, diets may not be consumed for a certain period after feeding took place (D'Abramo *et al.*, 1997), a stability of 1 to 4 hours has proven to be sufficient to produce good growth results. Binders resulting in higher stabilities may be more suitable in larger culture systems but their applicability will have to be examined in further experiments. The outcome of this experiment once more stresses the importance of careful preparation of crayfish feeds in addition to nutritional balance. Factors that principally influence the effectiveness of a diet have to be determined in advance and interactions of ingredients have to be continuously investigated.

8 Effects of Cyclop-eeze as an additional attractant and nutritional supplement in artificial diets on growth of juvenile signal crayfish, *Pacifastacus leniusculus* (Dana)

8.1 Introduction

After several experiments dealing with the effects of diet composition and form, the results have shown that the acceptance of artificial diets by crayfish in aquaculture must be increased to ensure good growth and food conversion rates (Ackefors *et al.*, 1992; D'Abramo *et al.*, 1997). Crayfish are slow and selective feeders. They are often described as omnivores and certainly their natural diet includes a wide variety of food sources. In aquaculture, however, crayfish feeding turns out to be far more difficult (Jussila, *pers. com.*). In intensive commercial farming, animals can no longer rely on natural food sources and improved artificial diets are of major importance (Ackefors *et al.*, 1992; Ackefors *et al.*, 1994). Feeds available for crayfish have succeeded in supporting good survival and satisfactory growth (Ackefors *et al.*, 1992; Wolf, 2000). However, food conversion rates are still considerably poor. The direct measurement of food conversion rates is still poorly developed for crayfish due to the inability to directly measure food consumption. Faeces sampling occurs to be very complicated and the measurements of feed intake are still being developed (Ahvenharju, *pers. com., unpublished data*). The amount of food loss, however, can be assessed more easily and it has to be concluded that crayfish feeding seems to be very inefficient. Due to the differences in physiology, ecology and aspects like feeding behaviour, comparisons between finfish and crayfish regarding growth, food conversion ratio and intensified farming are obsolete and crayfish will probably never be as easily farmable as many finfish species. Though, economical and ecological aspects cannot be ignored and food loss should be minimised in crayfish farming for both reasons.

As there are no readily available solutions for these problems, one attempt is to improve diets' abilities to stimulate animals' appetite and increase their feed intake. While crayfish diets have fulfilled certain criteria like being stable in water without influencing the animals' digestive system (*Chapter 7*), the next step in the development of crayfish feeds is to shorten feeding times resulting in less food loss.

Crustaceans as most aquatic animals use chemical signals to identify and orient towards prey and food sources. The importance of feeding stimulants in improving initial respondent behaviour as well as increasing overall feeding rate leading to reduce wasted feed is fully recognised (Lee and Meyers, 1997) and many studies have been carried out, emphasizing the

biology of chemoreception in crustaceans (D'Abramo *et al.*, 1997). It is also known that the commercial value of diets is very much dependent on attraction and feeding stimulation. Studies on chemoattractants for freshwater crayfish are rare and the use of attractants has primarily been limited to the development of trapping baits (Lee and Meyers, 1997). Therefore, experiments dealing with any kind of attractant can only have demonstrative nature. Cyclop-eeze (Lieberman, 2002), high in carotenoids and betaine, was chosen as a test attractant for this study to demonstrate the potential of stimulating chemoreception. Both, betaine and carotenoids are widely used as attractants in the production of fish diets and baits. Using a supplement containing both these attractants seemed therefore suitable for the purpose of this experiment. Selecting a product containing a range of unspecified ingredients was an attempt to bring the entire aspect of attractants into the spotlight. The results of this experiment are not meant to support a specific favorite product but to open the door and encourage more specific research in this area.

8.2 Cyclop-eeze



Picture 9: Range of Cyclop-eeze products as presented on the company's website. Argent Chemical Laboratories.

Cyclop-eeze (Picture 9) is a commercially available product marketed by Argent Chemical Laboratories. Its actual purpose is the replacement of Artemia, Spirulina and artificial plankton as starter diets for larval fish and shrimp. The manufacturer describes Cyclop-eeze as a superior

natural product, suitable for almost any aquaculture purpose. The copepods the product consists of “are a selectively bred, biologically engineered micro organisms which are cultured in a pristine arctic lake”. The decapods harvested for the production of the product are described to be of “blood orange” coloration. The company explains this with the high level of astaxanthin, an essential carotenoid for crustaceans’ coloration and metabolism. The importance of carotenoids in crayfish diets has been stressed by several authors (Ackefors *et al.*, 1992; D’Abramo *et al.*, 1997; Cuzon *et al.*, 1994) working on the field of crustacean nutrition. In addition to the high content of carotenoids, Argent stresses the unusual high levels of highly unsaturated fatty acids (HUFA) found in the copepods the product is made of. All polyunsaturated fats are increasingly recognized as important to animal health. Key omega-3 fatty acids include eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), both found primarily in oily cold-water fish. The importance of HUFAs has been stated by several authors (Blair *et al.*, 2001; Celada *et al.*, 1989; Curé *et al.*, 1995; Kontara *et al.*, 1995). High levels of omega-3 HUFA have been described as favourable for larval aquaculture applications and typically are obtained by food supplements (Naessens *et al.*, 1995). Cyclop-eeze is also seen an interesting product because it occurs to be rich in the attractant betaine as well as other palatability factors. Betaine as well as carotenoids and phospholipids are widely used as attractants and stimulants (D’Abramo *et al.*, 1997) but the amount of research carried out on crayfish is small. Unfortunately, research on Cyclop-eeze has not been published to a higher extend. The information available on nutritional values and compositional aspects of Cyclop-eeze are therefore limited to the manufacturer’s information and no independent research has been conducted evaluating the product in crustacean farming.

More detailed information about Cyclop-eeze are given in the tables provided by the manufacturer (Table 14):

Nutrient	Cyclop-eeze	
Protein %	59	
Lipid %	35	
	Omega-3 fatty acids (as percentage of total lipids)	
	18:3 linolic acid	10.45
	20:5 (EPA)	11.74
	22:6 (DHA)	11.09
Carbohydrate %	3	
Ash %	3	
Astaxanthin ppm	3000 to 7500	

Table 14: Effects of Cyclop-eeze. Nutritional composition and omega-3 fatty acid content of Cyclop-eeze. Argent Chemical Laboratories

8.3 Experimental set-up

For this experiment three diets were chosen for comparison. The first diet was produced from the same raw ingredients used earlier for this study. Wheat-starch (31.0%), fishmeal (54.0%) and fish oil (2.5%) as well as a base mix (12.5%) containing vitamins, carotenoids, minerals and Finnfix (2.0%) as a binding agent were mixed with purified water to form a dough that was then pelletised and dried in an oven at a constant temperature of 30°C for 48 hours. Calculations of the actual composition resulted in a distribution of macronutrients of 38.8% protein, 7.6% fat, 27.8% carbohydrates and fibre, 6.0% ash and 7.3% water.

The second diet used in this trial was prepared from the same ingredients, but an additional 0.5% of Cyclop-eeze replaced 0.5% of the fishmeal used for the diet. The small amount of Cyclop was chosen not to alter the composition of the two diets thus ruling out any effects due to macronutritional differences. Additionally, the intensity of attractants plays an important role in chemoreception and the experiment was designed to demonstrate if there are any effects of the attractant. High amounts of attractants would have biased the outcome due to their strong influence on the animals' chemoreception in all tanks of the system.

Raw ingredients for the Cyclop-eeze diet summed up as follows: w heat-starch (31.0%), fishmeal (53.5%), Cyclop-eeze (0.5%) and fish oil (2.5%) as well as a base mix (12.5%) resulting in 38.5% protein, 7.5% fat, 27.8% carbohydrates and fibre, 5.9% ash and 7.3% water. Again, the raw ingredients were mixed with purified water and - after pelletising the dough - the diet was dried in an oven at 30°C for 48 hours.

Frozen pieces of roach formed the third diet in this experiment. Roaches were chopped to small pieces (0.5-1.0cm) and portions of 3.5 to 4.5g were kept in the freezer to ensure the diet stayed fresh.

Nutritional values for roach were taken from Berg (1986): 17.5% protein, 3.0% fat, 4.5% ash and 75.0% water.

15 tanks of the experimental system were randomly chosen for this experiment and each tank was stocked with 20 animals resulting in an initial stocking density of 40 animals/m². The tanks were randomly divided into three treatment groups and animals were given two weeks time for acclimatisation before the experiment.

Measuring and weighing the animals started the three months experimental period. Animals were fed on daily basis except weekends and the amount of food given was 5% (on as-fed basis) of the initial body weight. Feeding amounts were not changed during the experiment.

The system was run in semi-open recirculation with water temperatures within 17.5 and 19.0°C and light conditions were set to an 18L:6D photoperiod by artificial illumination. Uneaten food, exuviae and dead animals were removed from the tanks daily and frequent cleaning of the tanks prevented fouling in the substrate of the tanks.

After three months, measuring and weighing the animals ended the experiment. All animals were killed by deep-freezing and freeze-drying the animals resulted in measurements of dry weight.

Calculation of treatment-based data was carried out by pooling the tank-based results of the measurements.

Statistics were carried out using the “R” language (The R Foundation of Statistical Computing, 2003) with the help of general linear models.

8.4 Results

The animals' initial carapace length ranged from 19.50 to 20.49mm and weight from 1.49 to 1.78g.

Carapace length at the end of the experiment reached from 25.14 to 29.11mm, final wet weights varied between 5.03 and 8.35g.

Mean results of the experiment are presented in *Table 15*:

Measurements	Diets		
	Fish	Cyclop	Basic
Initial carapace length (mm)	19.92	20.10	19.81
SD	0.27	0.36	0.37
Skewness	1.09	-0.26	1.61
Kurtosis	0.51	-2.14	2.48
Initial wet weight (g)	1.59	1.66	1.57
SD	0.06	0.09	0.09
Skewness	1.67	-0.11	0.71
Kurtosis	2.95	-0.11	-1.95
Final carapace length (mm)	27.20	27.24	25.77
SD	0.77	1.52	0.73
Skewness	-0.53	0.57	1.42
Kurtosis	-1.89	-2.88	1.80
Final wet weight (g)	6.18	6.44	5.42
SD	0.63	1.18	0.39
Skewness	0.56	1.26	0.15
Kurtosis	-2.74	1.85	-2.95
Carapace length increment (mm)	7.26	7.15	5.97
SD	0.84	1.55	0.89
Skewness	-0.07	0.38	0.90
Kurtosis	-0.43	-3.08	1.86
Wet weight gain (g)	4.59	4.78	3.85
SD	0.62	0.52	1.16
Skewness	0.65	-0.02	0.63
Kurtosis	-2.04	-1.79	-0.24
Dry Matter %	22.71	27.81	30.91
SD	1.04	6.08	8.51
Skewness	0.91	0.92	0.38
Kurtosis	-0.93	0.32	-1.64

Table 15: Effects of Cyclop-eeze. Mean values standard deviations, skewness and kurtosis for the measurements carried out in this experiment. The values represent the means of different treatment groups. For all treatments N = 5 (x 20).

Carapace length increment was 7.25mm for crayfish fed on fish, 7.15mm for those fed on the cyclop feed and 5.97mm for the animals fed on the basic diet (*Table 15, Figures 27, 28*).

For carapace length increment no statistically significant influence of the three different diets was found ($p = 0.1798$ and $F = 1.9862$). Taking the basic diet (5.97mm) as a baseline, effect sizes for carapace length increase were 1.18mm for the cyclop diet and 1.30mm for fish feeders, i.e. 19.8 and 21.8% higher growth, respectively. The residual standard error was 1.139 with 12 degrees.

Wet weight gain was 4.59g for the animals fed with fish, animals fed with the cyclop diet gained 4.78g and crayfish fed on basic diet gained 3.85g.

Analysis of the gain in wet weight did not reveal any statistically significant difference between the cyclop-diet and the other diets used ($p = 0.18$ and $F = 1.96$).

Basic diet (3.85g) served as a baseline and effect sizes were 0.92g for cyclop diet and 0.73g for fish i.e. 23.9 and 19.0% higher weight gain, respectively. The residual standard error was 0.78 with 12 degrees of freedom.

Once more, it seems that although the number of replicates in this experiment was high, the statistical power of this was insufficient.

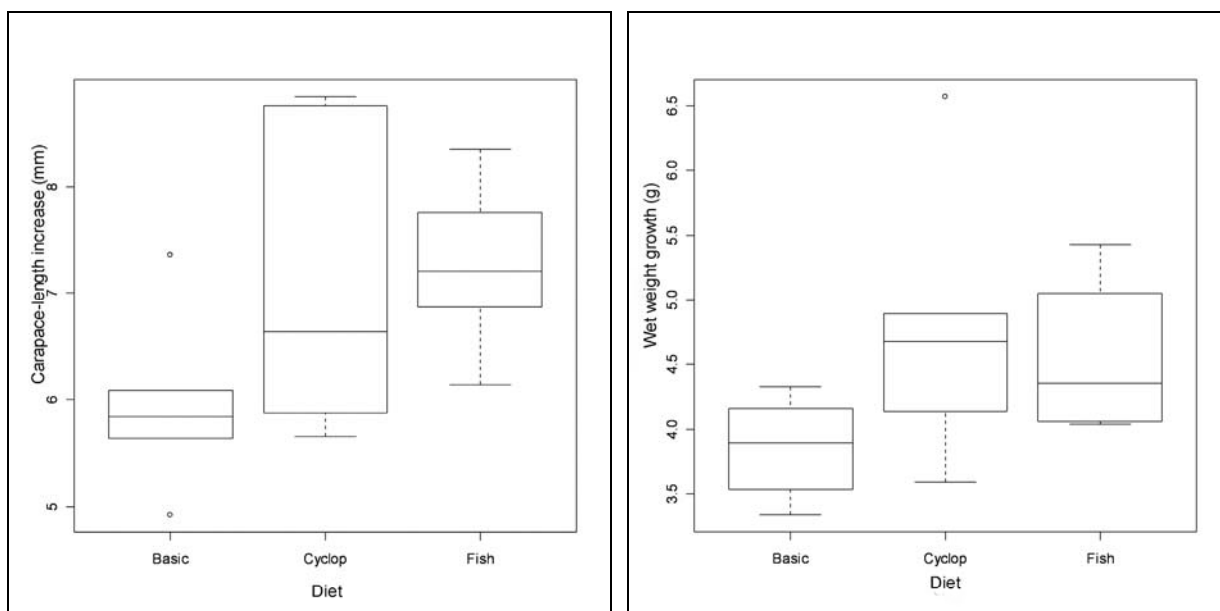


Figure 27 (left): Effects of Cyclop-eeze. Resulting increase in carapace length (mm) dependent on the diets fed to the animals. Treatment data were calculated from pooled tank data $N=5$ for all measurements. (Basic, Cyclop, Fish).

Figure 28 (right): Effects of Cyclop-eeze. Growth in terms of wet weight in g. Treatment data were calculated from pooled tank data $N=5$ for all measurements. (Basic, Cyclop, Fish).

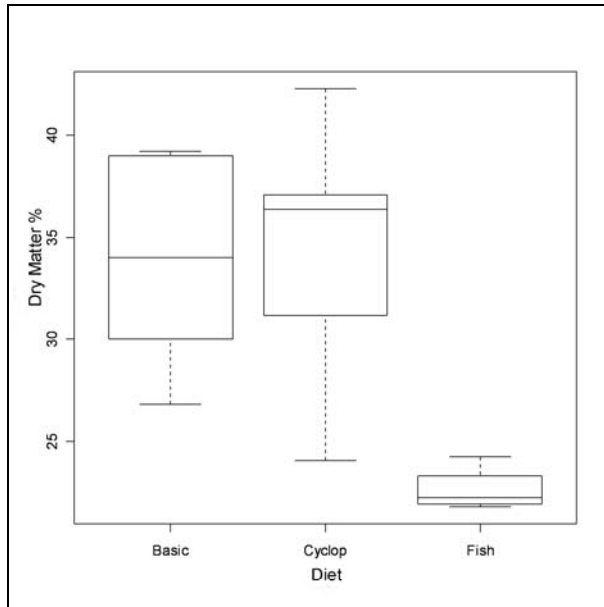


Figure 29: Effects of Cyclop-eeze. Outcome of the experiment in terms of dry matter contents (%). Data were calculated from pooled tank data, $N=5$ for all measurements. (Basic, Cyclop, Fish).

After freeze-drying, the dry matter contents of the animals was determined ranging from 21.8% to 42.3% with a mean of 30.2%. Statistics for the dry matter contents were carried out using general linear models. The analyses revealed a significant effect of the different diets on the animals' final dry matter contents ($p = 0.0058$ and $F = 8.15$). Effect sizes taking the basic diet (33.8%) as a baseline were 0.38% for cyclop diet and -11.10% for fish. The calculated residual standard error was 5.11 with 12 degrees of freedom.

Diet dependent results are given in *Table 15* and *Figure 29*.

8.5 Discussion

The decision to include this short-term experiment into the thesis was based upon the outcome of the other experiments. The development of suitable diets for crayfish culture has to take into account that crustaceans mostly rely on chemoreception when orientating towards food sources. Even though it remains uncertain whether the concentration or the kinds of attractants were insufficient or the experimental period was too short, a significant improvement in terms of growth was not visible. The enrichment of the diets with an attractant, sun-dried copepods with high levels of highly unsaturated fatty acids, biological pigments and immunostimulating biochemicals, did not seem to have any remarkable influence on the growth in terms of weight and length in this experiment. Animals fed with fish gained 4.6g during the experiment, those fed on the basic diet gained 3.9g and the wet weight gain for crayfish fed the enriched cyclop diet was 4.8g.

Attractants are known to influence the acceptance of diets by fish and crustaceans (Lee and Meyers, 1997; Holdich, 2001). The reception of chemicals released from the dietary source strongly influences the effects of a particular diet. A deeper investigation of the role attractants play in crayfish nutrition might therefore help to improve existing feeds and allow diets to be designed more specifically to meet the nutritional requirements of freshwater crayfish.

The complexity of the subject certainly cannot be assessed with a single experiment and the limitation to one attractant does not allow further conclusions to be drawn from the results of this test. The huge differences in dry matter content however may not be directly related to the attractants added to the diet. Both artificial diets resulted in very similar dry matter content (27.8% and 30.9%). The treatment group fed with fish returned a significantly lower dry matter content of 22.7%. These differences can be explained by the different moulting stages of the animals in different treatment groups. Observations of the experimental system have shown that the animals fed with the artificial diets moulted between 3 and 5 days earlier than the animals fed with fish. The increased water content of the fish feeders can therefore be explained by their stage of postmoult at the termination of the experiment. During moult, the animals' water intake is significantly increased and replacement of additional water with tissue growth takes place during the postmoult period. The results in terms of dry matter content suggest therefore a decrease in moulting frequency for animals fed with fish compared to those fed on artificial diets. The high wet weight of the animals fed on fish can also be explained with the high levels

of water in their bodies. The results of the experiment suggest that animals fed solely on fish grow slower than animals fed on balanced artificial diets. These results support the outcome of the experiment described in *Chapter 5* and suggest that roach has to be seen as an insufficient source of nutrients for signal crayfish.

9 General discussion

In fish culture, several biological and chemical processes are successfully used to eliminate metabolites from the water and improve production in intensive recirculation systems (Rosenthal and Otte, 1979). The experimental system used in the present work was therefore designed to include mechanical and biological filtration and water exchange could successfully be limited to 6.25% of the total water volume per day. The size of the tank system was an additional reason for good growth and low mortality during the experiments. By increasing the size of the water volume, an additional buffer for inhibiting and toxic metabolites was provided. Jussila (1997) reports similar findings suggesting high water volumes for intensified marron culture.

Frequent cleaning and daily removal of waste and exuviae or carrion was necessary for stable conditions and suitable water quality. The draining pipes from the tanks were designed to prevent animals from escaping but also provided difficulties in cleaning and maintenance. Additional problems were caused by the poor design of the mechanical filtration devices that were less efficient than planned and had to be cleaned too often.

Juvenile animals were selected for the experiments to utilize their high moulting frequency. Most animals moulted successfully once or twice during the experimental periods. However, as differences in growth rates were fairly insignificant, it has to be assumed that the feasibility for short-term experiments of less than 6 months is very limited. Due to their typical life cycle, crayfish growth does not show significant responses to different treatments in short- or mid-term studies.

These results also stress the weakness of traditional methods of growth measurement. The lack of differences in body weight or carapace length can easily lead to misinterpretations of experimental results if measurements do not include any kind of body composition responses. The measurement of body composition is certainly a valuable additional method of crayfish growth measurement. Investigations of body protein and lipid can provide essential information about the animals' response to different treatments (*Chapter 5*) and conventional methods of growth measurements are easily biased by the life cycle of crustaceans. Additional recordings of dry matter content help to detect differences in the moulting stages of experimental animals (*Chapter 8*) and can provide necessary extra information about the animals' status.

Signal crayfish (*Pacifastacus leniusculus*) fed with high protein diets grew better and their body composition seemed more balanced than animals fed diets with high lipid levels but the effects measured were overall small implying the omnivorous nature of the crayfish.

The type of food affected the body composition and showed patterns of conformation. Crayfish are strongly influenced by the lipid contents of their diet (D'Abramo and Conklin, 1985; Goddard, 1988; Fotedar *et al.* 1997), and the present work has confirmed earlier findings summarised by D'Abramo *et al.* (1997) in that high protein levels have inhibitory influence on the animals' growth.

In general, artificial diets, as the only sources of nutrition, have shown their potential resulting in good growth rates and body composition.

On the other hand, improvements in diet preparation leave certainly room for future research. The high applicability of optimised diets resulting from the response surface experiment has led to the development of a well-balanced experimental diet supporting good growth and survival of the animals. The high number of experimental tanks required and the complex design of the methodology are certainly weaknesses but they are outperformed by the applicability of the tested diets and its comprehensibility.

Food stability has proven less important than expected. Leaching and lack of stability of the diets has been a problem in crayfish research for a long time (D'Abramo *et al.*, 1997; Jussila, 1997) but the experimental results stress the importance of binding agents' inertness. Diets offering best digestibility can support good growth results without providing highest water stability. Different reasons may explain the failures of other binding agents to support good growth, but the similarity of a cellulose-based binder like Finnfix and the cellulose contained in the plant material many crayfish naturally feed on might be an explanation for the of this binding agent's suitability as earlier studies (Figueiredo *et al.*, 2001) have found cellulase and laminarinase activities in crayfish.

The poor growth of animals fed with alginate bound diets has been described earlier (*Chapter 7*) and can be explained by the influences of alginate binders on protein availability (D'Abramo *et al.*, 1997; Jussila, 1997).

Interestingly, the animals fed on the Keltone-bound diet had a remarkably high RNA/DNA ratio. One possible explanation might be the influence of nutrient availability on the animals' moulting cycle. The high RNA/DNA ratios measured agree with results published by El Haj *et al.* (1996). Rates of protein synthesis and RNA contents were elevated during the premoult period of *Homarus americanus*. This indicates that elevations in protein synthesis rates in different moulting stages could easily explain the high RNA/DNA ratios measured.

The experiments using the measurement of RNA/DNA ratio have proven the applicability of this new method in crayfish research. Earlier studies by Edsman *et al.* (1994) have been supported by the outcome of these experiments and the establishment of this new method allows a different approach to crayfish growth measurement. Further developments in sampling and possible refining of the chosen method have to take place to enable non-lethal sampling of crayfish taken from rearing and aquaculture facilities as from the wild.

More research is clearly needed regarding the effect of moulting cycle variation in RNA/DNA ratios before the results of the present study can be applied to natural populations or animals of different ages or size classes. However, in an experimental situation in which all the animals are approximately of same age, weight and size, the general picture emerging is that the RNA/DNA ratio is a good predictor of growth rate. The relationship found in this study between RNA/DNA ratio and feeding frequency cannot be uncritically applied without further calibration, which should take into account the effects of species, life stage, moulting cycle and temperature. In addition, the generality of the RNA/DNA ratio as an indicator of animal growth should be further considered as the method has returned misleading results like in the experiments on binding agents described in *Chapter 7*. In addition, re-calibration of the method when applying it to a different species has proven to be problematic and time consuming. Working with crayfish has caused additional difficulties because of the high enzymatic activity, which leads to rapid decomposing of dead animals and makes sampling and handling of the samples extremely difficult.

The use of the method in addition to somatic growth rate measurements is justified despite its potential problems. If the RNA/DNA ratio measurements can be calibrated to species, status and environmental factors, it can provide a snapshot index of crayfish growth rate under natural conditions, which makes time consuming incubations unnecessary provided their phase in the moulting cycle could also be determined. Methods for the determination of the phase in moulting cycle have been developed and Ahvenharju (1998) has described a non-invasive method using X-ray for the investigation of the crayfish gastroliths.

The growth rate observed in such experimental incubations can be biased by experimental artefacts, which are avoided using the RNA/DNA method. Second, the rapid response (i.e., from a few hours to several days rather than weeks or months which are required for measuring biomass increments) in the RNA/DNA ratio to changes in either food quality or food quantity significantly reduces the problems associated with diet development and diet composition experiments.

When measuring the dry matter contents of the experimental animals in some of the experiments, the animals showed high synchronism in moulting as fairly constant dry matter contents were measured within the experiments. When moulting, the animals have to take up large amounts of water to enlarge their soft new exoskeleton. In the time after ecdysis the animals use water as replacement for the body tissue they have to build up after the new exoskeleton has entirely hardened (Lee and Wickins, 1992) and increased water content can thus be seen as an evidence of recent moulting.

Similar synchronism like found in this study has also been reported by other authors (Jonsson and Edsman, 1998) and can be seen as a normal occurrence even in constant laboratory environments although the actual mechanisms behind the synchronisation are not yet known. The experiment about the chemoattractants described in *Chapter 8* certainly has shown an exception from this synchronism. The delay of moulting due to differences in nutritional supply has been described by other authors before (Jussila, 1997, Taugbøl *et al.*, 1989; Taugbøl and Skurdal, 1992) and the regulation of crayfish moulting cycles is still poorly investigated. The assumed effects of diet type and chemoattractants could not be seen and growth rates were fairly similar for all diets. As omnivores, crayfish have shown to accept diets easily and pelletised diets did not show any disadvantages compared to block shaped feeds. Cleaning of the aquaculture system however is made significantly easier by the use of large feed blocks. Further developments have to focus on the development of improved feeders and diet types. Demand feeders might also be used as a tool for basic experiments and automatisation. Research tasks covering feeding behaviour and learning behaviour have to be included in the development of crayfish aquaculture.

While types of diets seem to have a minor importance in crayfish feeding, the failure of the chemoattractants could have several reasons. The concentration of the attractant used may have been too low and suitable amounts of chemoattractants have to be found before further experiments can focus on the favourable types of attractants. It also cannot be ruled out that the diets' own contents of carotenoids and phospholipids covered the actual effect of stimulants and attractants added to the diets. Diets were also high in fish meal, a known source of stimulatory amino acids. (Goddard, 1988)

However, differences in body composition were detected when animals were fed a control diet consisting of frozen roach. The findings agreed with the assumption of crayfishes' conformation reaction to increased dietary lipid contents and supported the need for balanced artificial diets since roach did not provide sufficient amounts of necessary macronutrients.

For future experiments, larger experimental systems would be preferably to increase the number of replicates used in the experiments. The usage of tank-based means was sufficient for the experiments carried out but increased numbers of replicates might help to minimise errors in measurement and support the results of the experiments. Most of the experiments were carried out with 4-5 replicates for each treatment and the results have shown that the number is insufficient. In addition to an increase in tank numbers or as an alternative, individuals could be monitored by marking the animals and following their individual development. Experiments with marked individuals successfully have been carried out earlier (Wolf, 2000) but the marking of high numbers of crayfish is extremely time-consuming and stressful for the animals. In addition, the used tags may lead to higher variations in the animals' body composition. Visible implant fluorescent elastomer tags (VIE) used in earlier studies (Godin *et al.*, 1996) can hardly be removed from the animals' tissue and the amounts of elastomer inserted into an animal cannot be precisely monitored.

An important result of this study is the successful rearing of juvenile signal crayfish with the developed artificial diets. The animals did not show any symptoms of deficiency. High survival and good growth were received without providing any additional sources of nutrients and important steps in the development of research methodology and diet formulation could be made.

This study has certainly improved available crayfish diets. Especially the mixture approach has resulted in a better understanding of macronutritional diet composition for astaciculture.

Applicability of the diets was one of the key factors this study dealt with. It was shown that artificial diets in general have good potential to be used as a replacement of natural food sources in intensified crayfish aquaculture.

Anyhow, results from the experiments carried out in this study suggest, dietary composition has still not reached the efficiency seen in other fields of aquaculture. Comparing the efficiency of crayfish feeds with the diets developed for finfish farming, it becomes obvious that diet development for freshwater crayfish is still far from optimal. Further research has therefore to be carried out.

10 Conclusions

It has been the goal of this study to gain important information about growth and the effects of macronutritional composition of diets on freshwater crayfish in aquaculture. Several key issues of this subject have been included in the experiments carried out for this project. The importance of taking into account several aspects of diet preparation and diet composition was clearly stressed by the results of this study.

The results of this study have increased the necessary knowledge about crayfish nutrition. Better diets are available and the introduction of new methodology requires further research in crayfish culture. The demonstration of the feasibility of new feeding experiment designs and the potential of stimulants in nutritional research are innovations that put the spotlight on important questions and might provide the baseline for future research.

The main conclusions from the present work are:

1. Mixture designs are well suited to feed formulation problems and can ensure that experimental and application spaces coincide, i.e. the results are readily applicable.
2. Measurements of body composition improve monitoring of different treatments by including the life cycle of crustaceans better than traditional methods.
3. RNA/DNA ratio measurement can be used in crayfish research. The method has been successfully established but limitations have also been detected. More research has to be carried out to finally evaluate the method.
4. Development of crayfish diets is still an important field for research. Optimising diets and deepening the understanding of nutritional requirements will be the next steps in research. Intensification of crayfish farming requires further improvements of artificial feeds.
5. The effects of binding agents have been shown and their importance in diet formulation has been stressed. Detailed investigation of the influences of binders on digestibility and feed intake requires further research.
6. Diet types play a minor role in crayfish nutrition. Pelletised diets will probably have best applicability and can easily be used in crayfish culture.

7. The role of attractants and stimulants is still widely undiscovered. Future research will have to focus on the understanding of chemoattractants in crayfish nutrition to improve feeding efficiency.

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11.2 Internet links and www pages

Analytical Techniques in Aquaculture Research (24.04.2004):

- <http://allserv.ugent.be/aquaculture/ATA/index.htm>

Biochemistry syllabus, Department of Chemistry and Biochemistry,
The University of Texas at Austin (08.05.2004)

- <http://www.cm.utexas.edu/CH369/Kitto/ReadSched2004.htm>

Centers for disease control and prevention, CDC (18.05.2004)

<http://www.cdc.gov/>

Crayfish Innovation Center (21.05.2004)

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Finnish Games and Fisheries Research (23.05.2004)

- <http://www.rktl.fi>

Hydralco Hydrocolloide GmbH (12.04.2003)

- <http://www.hydralco.de/index.htm>

International Specialty Products (ISP) (20.05.2004)

- <http://www.ispcorp.com/index.html>

The Plot page (22.05.2004)

- <http://dover.byu.edu/splustut/Statsci1/Chapter3/3LinReg3.htm>

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- <http://www.r-project.org/>

The R Projcet: Documentation and Help (22.05.2004)

- <http://stat.ethz.ch/R-manual/>

Willner Chemists, The Nutritional Supplements Professionals (03.05.2004)

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World Aquaculture (15.05.2004)

- <http://www.newmex.com/platinum/data/index2.html>

12 Summary

In Europe, increased attempts to intensify crayfish aquaculture have been carried out in recent years. Some of the major problems arising from the intensification are related to dietary supply of the animals in culture ponds and tanks. This study therefore focussed on some of the key objectives supporting the development of suitable diets for signal crayfish *Pacifastacus leniusculus* aquaculture.

A large-scale response surface experiment was carried out to study the influences of different macronutrients (protein, lipid, carbohydrate) on crayfish growth and body composition. The results from this experiment led to the development of an improved experimental diet containing high levels of protein and carbohydrates, while increased lipid concentrations seemed to be influencing growth adversely.

Further experiments were carried out to improve diet quality. For this purpose, diet types and the suitability of different binding agents were investigated. A small experiment was carried out to monitor the effects of attractants on growth of juvenile signal crayfish.

A second objective of this study was the establishment of a new method to measure physiological responses of crayfish to different treatments. Running mid-term or long term experiments require high amounts of resources and slow down the process of dietary development. The measurement of RNA/DNA ratio has proven to be a suitable instrument to examine short-term metabolic responses to different key factors such as feeding regime, diet quality and digestibility and bioavailability of essential nutrients.

The study has stressed the importance of proper diet preparation and composition for successful astaciculture. Binding agents were found to have major influence on diet digestibility and bioavailability of macronutrients. As another result of this study, further experiments are regarded to be necessary to examine the response of signal crayfish to different levels of lipid and protein in their diet. Some of the results obtained suggest a lack of regulatory capacity regarding the animals' protein/lipid ratio. Further experiments however will have to be carried out to proof this hypothesis.

13 Zusammenfassung

Versuche zur Intensivierung der Flusskrebszucht in Europa haben in den letzten Jahren deutlich an Bedeutung gewonnen. Einige der Hauptprobleme in diesem Zusammenhang sind eng mit der Entwicklung geeigneter Alleinfuttermittel für die intensive Aquakultur verknüpft.

Diese Arbeit konzentriert sich daher auf zwei wesentliche Aspekte der Krebszucht.

Zum einen sollen essentielle Fragen zur Futtermittelentwicklung beantwortet werden, zum anderen sollte eine passende Methode zur Analyse kurzfristiger Stoffwechselreaktionen etabliert werden.

Bisherige Experimente mit Signalkrebsen *Pacifastacus leniusculus* nehmen meist Gewichts- und Längenzuwachs in Abhängigkeit von verschiedenen Faktoren als Maßstab für das Wachstum der Tiere. Hierzu sind bislang mittel- bis langfristige Experimente notwendig. Die Einführung der Messung von RNS/DNS Verhältnissen hilft daher, Entwicklungszeiten zu verkürzen, da bereits kurzfristige Reaktionen auf Faktoren wie Futtermittelqualität, Verfügbarkeit der Nährstoffe und Verdaulichkeit der Futtermittel, sowie Fütterungsintervalle zukünftig ohne mehrmonatige Experimente abgeschätzt werden können.

Die Auswirkungen verschiedenster Futtermittelzusammensetzungen im Hinblick auf die wichtigsten Makro-Nährstoffe (Protein, Fett, Kohlenhydrate) wurde in einem groß angelegten Fütterungsversuch untersucht. Die Ergebnisse dieses Versuches führten zur Entwicklung besserer Versuchsfuttermittel, deren hoher Protein- und Kohlenhydrat-Anteil, so wie der gesenkte Lipidgehalt besser auf die Bedürfnisse der Tiere abgestimmt sind. Zudem wurden wichtige Aspekte wie Darreichungsform der Futtermittel, Zusatz von Lockstoffen und die Eignung verschiedener Bindemittel untersucht.

Die Ergebnisse dieser Versuche verdeutlichen die Wichtigkeit sorgfältiger Entwicklungsarbeit bei der Futtermittelherstellung. Die beobachteten Auswirkungen verschiedener Bindemittel auf die Verfügbarkeit von Nährstoffen und die Steigerung der Futterakzeptanz durch richtige Darreichungsformen und eventuelle Lockstoffzusätze sind ebenfalls wichtige Aspekte der Futtermittelentwicklung.

Weitere Untersuchungen werden sich zukünftig zudem mit der Regulation der Protein/Lipid-Balance bei Flusskrebsen beschäftigen müssen, da die Ergebnisse einiger Versuche eine mangelnde Regulationsfähigkeit der Tiere in diesem Bereich vermuten lässt.

14 Curriculum vitae

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Diploma-Thesis: “Effects of time of feeding on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus*“, Evo Fisheries Research Station (Finnish Games and Fisheries Research Institute (RKTL))

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University degree: “Diplom-Biologe”
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Job experience:

December 1991 – September 1994
Male nurse, psychiatric hospital
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01.03.1997 – 31.12.1998
Student research assistant GEOMAR – Institute for marine geo-
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Februar – März 1998
Research assistant onboard research vessel
“*FS Poseidon*“
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01.01.1999 – 30.04.1999
Student research assistant, Department of fisheries biology,
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Scholarships:

01.01.2001 – 31.12.2001
Grant by DAAD, German Academic Exchange Service

01.01.2002 – 31.12.2003
Grant by German National Academic Foundation (Studienstiftung
des deutschen Volkes)

Projects worked on:

July 1998

Schneider, Oliver and Wolf, Sebastian

„Betrachtung der Stickstofflast in einer marinen Kreislaufanlage, Biofiltrationsleistung in Abhängigkeit von Durchfluss, spezifischen Substratoberflächen und induzierter Ammoniumbelastung“ (“Monitoring of the nitrogen load in a marine recirculation aquaculture system depending on exchange rates, biofiltration, specific filtration media and Ammonia load”)
Department of fisheries biology, University of Kiel

March 2000

Wolf, Sebastian

“Effects of time of feeding on growth and body composition of juvenile signal crayfish, *Pacifastacus leniusculus*“
Diploma-Thesis, Department of fisheries biology, University of Kiel and Evo Fisheries Research Station (Finnish Games and Fisheries Research Institute (RKTL))

October 2000

Ruohonen, Kari and Wolf, Sebastian

„Kolmen rehun vertailu täpläravun kasvatuksessa“
 (“Effects of three different diets on growth of signal crayfish”)

Kala- ja riistaraportteja, Evo Fisheries Research Station (Finnish Games and Fisheries Research Institute (RKTL))

15 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die Arbeit hat noch keiner anderen Stelle im Rahmen eines Promotionsverfahrens vorgelegen und wurde weder veröffentlicht noch zur Veröffentlichung eingereicht.

Kiel, den