NOBLE CRAYFISH AQUACULTURE

EMBRYONIC DEVELOPMENT AND JUVENILE PERFORMANCE

Dissertation

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SUMMARY

Freshwater crayfish play an important role in freshwater ecosystems and represent a highly appreciated food item. The European crayfish fauna comprises five indigenous species and noble crayfish *Astacus astacus* (L. 1758) is the only species native to Northern Germany. Once widespread and common in almost any kind of freshwater habitat, the crayfish plague, the spread of non-indigenous crayfish species and physical as well as chemical degradation of natural water bodies greatly threaten our largest mobile invertebrate and have locally driven it to extinction.

Noble crayfish are produced in extensive and semi-intensive pond systems for restocking of natural water bodies and for consumption. Intensification of crayfish culture is challenging due to the cannibalistic behaviour, the low fecundity, the slow and intermittent growth and the seasonal growth pattern. A promising approach to an efficient production of table-size crayfish and stocking material is the extension of the animals' growth period in the first year by an accelerated incubation of eggs that is followed by a nursery phase until ambient conditions are suitable for pond stocking.

Intensification efforts of astacid culture depend on the appropriate assessment of the animal's physiological condition both for research and application. I could show that RNA/DNA ratio is a suitable parameter to assess overall growth capability in juvenile noble crayfish. Based on RNA/DNA ratios different feeding regimes were detected earlier than by morphometric growth parameters while RNA per wet weight failed to detect any differences at all. Furthermore, RNA/DNA ratio explained 89 % of the variation found in specific growth rate. The results are applicable likewise for the assessment of physiological condition of natural and cultured stocks and ease the development of commercial feeds by reducing the necessity for long experimental periods.

By using artificial incubation for an accelerated incubation of early stripped eggs of noble crayfish, I demonstrated that dormancy in this species represents a form of diapause that is regulated by temperature and can be accelerated beyond a threshold only at the expense of higher mortality and reduced juvenile fitness. A moderately long cold period of 6-7 weeks from gastrulation favoured high stage II survival and wet weight. Furthermore, this diapause period synchronised embryonic development and reduced hatching and moulting period to 7 days with no overlap between both. Applying this to culture, stage II juvenile crayfish can be available in late March which is an appropriate time point for the start of a nursery phase.

Suitable rearing conditions for juvenile crayfish were investigated in an on-farm study for two starting points (early: 28th of March, late: 21st of May) each followed by an eight-week nursery. Lighting is crucial for the entrainment of a nocturnal locomotor activity pattern thereby affecting performance. Therefore, the effects of natural sunlight and different artificial light sources on crayfish performance and periphyton characteristics were investigated. Survival was higher if crayfish were stocked later and natural sunlight yielded highest specific growth rates in both trials. The importance of naturally occurring food items differed between natural sunlight and the artificial light sources and periphyton characteristics partly explained differences in crayfish performance. This illustrates the potential of periphyton as a complementary food source in nursery of noble crayfish and shows that its growth can be promoted by novel lighting techniques.

The results obtained in my thesis favour the production of table-size crayfish within two summers, are of high value for the conservation of endangered stocks of indigenous astacid crayfish and deliver valuable information for the development of commercial feeds and novel approaches to juvenile rearing.

ZUSAMMENFASSUNG

Flusskrebse sind wichtige Schlüsselorganismen der benthischen Wirbellosenfauna in Süßwasserlebensräumen und gleichzeitig ein geschätztes Nahrungsmittel. In Europa kommen fünf einheimische Arten vor, darunter der Edelkrebs *Astacus astacus* (L. 1758) als einzige heimische Flusskrebsart in Schleswig-Holstein. Durch die Krebspest, die Ausbreitung invasiver gebietsfremder Flusskrebse sowie die Degradation der natürlichen Gewässer ist diese größte mobile Wirbellosenart stark gefährdet und lokal vom Aussterben bedroht.

Die Aufzucht von Flusskrebsen findet in extensiv und semi-intensiv bewirtschafteten Teichen statt und dient sowohl der Bereitstellung von Besatzkrebsen als auch der Produktion von Speisekrebsen. Eine Intensivierung ist aufgrund des kannibalistischen Verhaltens, der geringen Anzahl an Nachkommen sowie aufgrund des langsamen und saisonalen Wachstums problematisch. Ein vielversprechender Ansatz für eine effizientere Produktion ist die Verlängerung der Wachstumsphase im ersten Jahr. Dies kann durch die beschleunigte Erbrütung der Edelkrebseier gefolgt von der Aufzucht der juvenilen unter kontrollierten Bedingungen erreicht werden.

Eine zentrale Voraussetzung für die Intensivierung der Flusskrebs-Aquakultur ist die exakte Bestimmung der physiologischen Verfassung der Krebse. Ich konnte zeigen, dass das RNA/DNA Verhältnis hierfür ein geeigneter Parameter ist. Auf Basis des RNA/DNA Verhältnisses konnten Unterschiede in der Nahrungsverfügbarkeit schneller nachgewiesen werden als mit morphometrischen Methoden, während auf Basis des Verhältnisses von RNA zum Nassgewicht der Probe keine Unterschiede erkennbar waren. Zudem zeigt das RNA/DNA Verhältnis eine gute Korrelation (89 %) zur Wachstumsrate der Krebse. Diese Ergebnisse erlauben die Anwendung des RNA/DNA Verhältnisses zur Bestimmung der Kondition freilebender Bestände und solcher in Aquakultur. Zudem können sie die Entwicklung von kommerziellen Futtern für Flusskrebse erleichtern, da kürzere Versuchsdauern ermöglicht werden.

Mittels künstlicher Erbrütung früh abgestreifter Krebseier konnte ich zeigen, dass deren Ruhephase eine echte Diapause darstellt. Eine Verkürzung durch Temperaturerhöhung ist möglich, allerdings ist dies ab einer bestimmten Grenze mit erhöhter Mortalität und reduzierter Fitness der Juvenilen verbunden. Eine Kältephase von 6-7 Wochen ab dem Zeitpunkt der Gastrulation ist ausreichend, um hohe Überlebensraten und Schlupfgewichte sicher zu stellen. Zudem synchronisiert die Kälteperiode die Embryonalentwicklung und reduziert die Zeitspanne der Schlupfe und der ersten Häutungen auf je sieben Tage ohne dass es dabei zu

Überschneidungen kommt. Für die Anwendung in der Krebszucht bedeutet dies, dass Brütlinge zu Ende März bereitgestellt werden können, was wiederum ein geeigneter Zeitpunkt für den Beginn des Vorstreckens ist.

Während des Vorstreckens der juvenilen Krebse ist eine Beleuchtung der Becken wichtig, da Licht das tageszeitliche Aktivitätsmuster der Krebse beeinflusst und dieses sich wiederum auf Überlebensrate und Wachstum auswirkt. In zwei aufeinander folgenden Experimenten im Produktionsmaßstab wurde der Einfluss von natürlichem Sonnenlicht und künstlichen Lichtquellen bei der Aufzucht frisch geschlüpfter Krebse über einen Zeitraum von je acht Wochen untersucht. Dabei wurden die Krebse einmal am 28. März und einmal am 21. Mai besetzt. Die Überlebensrate war bei den später besetzten Tieren höher und unter natürlichem Sonnenlicht wurden immer die höchsten Überlebens- und Zuwachsraten festgestellt. Natürliche Nahrungsressourcen waren von unterschiedlicher Bedeutung wobei Unterschiede in Wachstum und Überlebensrate zum Teil auf Unterschiede in Biomasse und Zusammensetzung des Periphytons zurückgeführt werden konnten. Die Ergebnisse zeigen erstmals die potentielle Bedeutung von Periphyton für juvenile Edelkrebse und liefern Hinweise, wie dessen Wachstum mithilfe moderner Beleuchtungsmittel gefördert werden kann.

Die Ergebnisse dieser Arbeit erleichtern die Produktion von Speisekrebsen innerhalb von zwei Wachstumsperioden, sind bedeutend für die Erhaltung bedrohter einheimischer Flusskrebsbestände und stellen wertvolle Informationen für die Entwicklung künstlicher Futter sowie innovativer Aufzuchtmethoden für Flusskrebse bereit.

PREFACE

In Germany and adjacent countries, crayfish first attracted significant attention of zoologists during the early 18th century. This resulted in several scientific publications the majority of them dealing with noble crayfish *Astacus astacus* (L. 1758) and Haeckel's (1857) dissertation "Über die Gewebe des Flusskrebses" likely being the most widely distributed and acknowledged from that time. A few years later, however, Huxley (1880) has summarised the upcoming importance of crayfish for science most appropriate:

"*I have desired, in fact, to show how the careful study of one of the commonest and most insignificant animals, leads us, step by step, from every-day knowledge to the widest generalizations and the most difficult problems of zoology; and, indeed, of biological science in general*".

With little less pathos, embryonic development of noble crayfish *A. astacus* was first characterised and illustrated by Rathke (1829), followed by Reichenbach (1877; 1886) who continued to work on this aspect of crayfish biology and described in detail the development from the blastoderm to the first juvenile stages. In particular, Reichenbach (1886) breaks down the embryonic development to 12 developmental stages and pays particular attention to the crayfish nervous system – studies of the latter are of great importance in recent neuroscience (e.g. Huber et al. 2011). Furthermore, Reichenbach (1886) contains impressive drawings that everyone interested in this aspect of crayfish or decapod development biology should notice. Approximately fifty years later, Zehnder (1934) completed the preceding studies by Rathke (1829), Lereboullet (1862) and Reichenbach (1877; 1886) by describing a gradual staging system of embryonic development for noble crayfish that includes 15 consecutive developmental stages. He assigned the respective animals' age to the stages that are for the first time illustrated by drawings and photographs. The staging system established by Zehnder (1934) still is the basis for more recent studies (Celada et al. 1987; Sandeman and Sandeman 1991) that have also been used for this thesis.

The first comprehensive publication on "Pflege und Fang" of crayfish was presented by Dröscher (1906). Likely, the increasing demand of knowledge on fishing for and culture of crayfish was caused by the drastic decline of natural stocks in Europe caused by the appearance of crayfish plague *Aphanomyces astaci* (Schikora, 1906) in the mid-70s of the late 19th century. Dröscher (1906) additionally provided one of the rarely accessible proofs for the economic importance of crayfish trade around 1900. Between 1893 and 1904 Germany imported approximately 1100

tonnes of crayfish worth 1.5 million marks, the majority originating from Russia. During the same period, 500 tonnes worth 866 million marks were exported primarily to France. Thereby, hundreds of millions of crayfish have been displaced from east to west – despite this, recent studies on population genetics of noble crayfish (e.g. Schrimpf et al. 2011; 2014; 2017; Schmidt et al. 2015) are able to detect clear patterns in population genetic structure and identify populations of high conservation interest. Following Dröscher (1906), Schikora (1916) published a, what we would call today, *best-practise handbook* that provided information on the current state of knowledge of restocking noble crayfish in natural waters to the general public. This somehow constituted a first call for a nation-wide effort of species conservation for noble crayfish.

Between 1945 and 1970, crayfish served science predominantly as a model organism in neuroscience and physiology producing several highly cited publications in the field (e.g. Scudamore 1947; Furshpan and Potter 1959). In 1972 the International Association of Astacology was founded and since 1977, publishes a peer-reviewed scientific journal covering all aspects of crayfish biology from various disciplines. In the first issues, studies on crayfish culture were well represented as, from the 1970s until the late 90s, culture of crayfish gained strong attention worldwide (e.g. Holdich 1993; Jones 1995a; 1995b). In Europe, various aspects of cold-water crayfish culture were investigated (Reynolds et al. 1992; Taugbøl and Skurdal 1989; 1990; Ackefors 2000) since the continuous spread of invasive crayfish hindered the recovery of native crayfish populations (Bohman et al. 2006) and frequently did not result in harvestable stocks of non-indigenous species (Sandström et al. 2014).

During the last two decades, more thousands of original research papers that dealt with crayfish have been published covering a wide range of scientific disciplines from freshwater science (e.g. Gherardi and Acquistapace 2007) to – from a crayfish point of view at least at first glance – more exotic field of psychology (Jimenez-Morales et al. 2018). However, this most recent period of the crayfish way through science can well be assigned to the study of their ecology and conservation (Gherardi 2011; Pârvulescu et al. 2020). Commercial culture of noble crayfish in Europe has, however, failed to establish on a large scale and today primarily serves restocking purpose, while culture of warm-water crayfish species for consumption (i.e. *Procambarus clarkii*, *Cherax* spp.) recently caught up to the “golden age” of crayfish culture in the 1980s and 1990s (Garca de Yta 2016; Jones and Valverde 2020; Rigg et al. 2020).

Bearing this knowledge in mind that has accumulated through almost 200 years of research this thesis aims to contribute to the sustainable use and conservation of noble crayfish – a species that has long been, still is and hopefully will be of tremendous importance for zoological

science, for the ecology of freshwater ecosystems and for human pleasure. Therefore, I studied some aspects of noble crayfish biology that I consider crucial for a successful culture and I hope that my findings promote the implementation of a nursery phase in production of noble crayfish, thereby facilitating an economically viable culture that supports the establishment of successful restocking programs.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Systematics and distribution of freshwater crayfish

"… if the West American and Amur-Japanese crayfish switched their places, everything would suddenly make more sense" (Birštejn 1985, cited in Ďuriš and Petrusek 2015).

Within the Arthropoda, freshwater crayfish are decapod Malacostraca, belong to the suborder Pleocyemata (decapods taking care of eggs, also of the first stages of the juveniles) and are members of the infraorder Astacidea (Ďuriš 2015). The taxon Astacidea, sometimes also referred to as Astacida or Astacoida, comprises four recent superfamilies: Enoplometopoidea (de Saint Laurent, 1988; reef lobsters) and Nephropoidea (Dana, 1852; true lobsters) with exclusively marine species and Astacoidea (Latreille, 1802) and Parastacoidea (Huxley, 1879) with exclusively freshwater crayfish. It is well accepted that freshwater crayfish are a monophyletic group (Scholtz 1999; De Grave et al. 2009) and their adaptation to their freshwater habitat has most likely only happened once approximately 200 million years ago (Scholtz 1999). Today, there are more than 650 recognised species of crayfish assigned to 38 genera worldwide (Ďuriš 2015; Crandall and De Grave 2017). The number of accepted species is, however, continuously increasing due to description of new species, in particular from North (e.g. Bloom et al. 2019) and South America (e.g. Ribeiro et al. 2016), and the application of molecular methods that helps unravelling cryptic species (e.g. Maguire et al. 2014).

Crayfish systematics, in particular at the family and genus level, is up to continuous debate (De Grave 2009; Kouba et al. 2014; Crandall and De Grave 2017). A long-time accepted system further divided the Astacoidea into the families Astacidae and Cambaridae while the Parastacoidea consist of a single family, the Parastacidae. It can be safely stated that Astacidae and Cambaridae are naturally present in the northern hemisphere while Parastacidae occur in the southern hemisphere (Scholtz 2002). A very intrusive pattern in the northern hemisphere Astacoidea is the occurrence of Astacidae in Europe and Asia and the restriction of Cambaridae to North America. This pattern is, however, disrupted by the presence of *Pacifastacus* on the Pacific coast of North America and *Cambaroides* in the Far East of Asia. There is currently no widely accepted explanation for the presence of *Pacifastacus* and *Cambaroides* separated from the diversity centres of their families. A particular problem is that striking morphologic characters (e.g. presence/absence of spermatheca) and results of phylogenetic studies currently do not

match the existing families of Astacidae and Cambaridae. The introduction of two new families has been discussed (Ďuriš 2015) as well as the inclusion of *Cambaroides* in the Astacidae (Bracken-Grissom et al. 2014) and recently, Crandall and De Grave (2017) placed *Cambaroides* in the newly established family Cambaroididae while *Pacifastacus* remains a member of the Astacidae. Despite their recently updated classification there are still several unresolved problems in systematics of freshwater crayfish, in particular the monophyly of northern hemisphere family Astacidae and of several genera in the Cambaridae is questionable (Scholtz 1999; Crandall and De Grave 2017).

Freshwater crayfish are present on all continents except the Antarctic and are also absent from continental Africa (Hobbs 1988). There are two distinct centres of diversity, one in the south-eastern United States and one in southern Australia (Ďuriš 2015). The North American genera *Cambarus*, *Procambarus* and *Orconectes* (Cambaridae) account for more than 50 percent and Southern hemisphere *Cherax*, *Engaeus* and *Euastacus* (Parastacidae) for another 20 percent of global species richness (Crandall and De Grave 2017).

The native crayfish fauna of western and northern Europe comprises only five species in two genera (*Austropotamobius* and *Astacus*) that all belong to the family Astacidae (Füreder 2016). *Austropotamobius pallipes* is considered a complex of at least two species (*A. pallipes* and *A. italicus*) and shows a more western distribution in Great Britain, France, Spain, Italy and Croatia while *Austropotamobius torrentium* (Schrank, 1803) occurs from central Germany to the Balkan peninsula (Kouba et al. 2014). *Astacus leptodactylus* s.l. is as well regarded as a complex of at least to species and sometimes also referred to as *Pontastacus leptodactylus* (Śmietana et al. 2006). The native range of *A. leptodactylus* ranges from the Ural in the east to the Balkan peninsula in the west (Kouba et al. 2014) but as it is of interest for fisheries and aquaculture, *A. leptodactylus* has been translocated to central and western Europe where it established populations, predominantly in lotic waters. *Astacus pachypus* (Rathke, 1837) has a very small natural range and can be found only at the northern and eastern parts of the Black Sea and around the Caspian Sea (Kouba et al. 2014). Interestingly, this colourful crayfish species is mesohaline and found in brackish and freshwater habitats (Skurdal and Taugbøl 2002). The natural range of noble crayfish *A. astacus* (L. 1758) extends from the Baltic states over the southern areas of Scandinavia to France in the west and the Balkan peninsula in the southeast. The highest genetic diversity of noble crayfish was preserved in the Balkan peninsula (Schrimpf et al. 2014) but local hot spots of high genetic diversity also exist elsewhere, in particular in northern Germany (Schmidt et al. 2015; Füreder 2016). For those populations, the establishment of „arc-sites“ is vital to the conservation of the species (Kozák et al. 2011). In particular, gravel pit waters are suitable as secondary

habitats for noble crayfish but restocking programs require, however, the effective production of juveniles from wild populations (Keller 2009, Kouba 2011).

Biology of freshwater crayfish

"*Auch ist wohl mancher Mißerfolg durch Unkenntnis der Lebensbedingungen des Krebses und Nichtbeachtung der zu seiner rationellen Pflege unentbehrlichen Maßnahmen verursacht worden*" (Dröscher 1906)

Knowledge on an animals' life cycle as well as on biotic and abiotic parameters influencing its foraging behaviour, activity patterns, growth and reproduction are crucial for successful culture. General patterns of foraging and growth and, as a results of an adaptation to freshwater, numerous characters in their life-cycle and mode of reproduction are similar in all crayfish species (Scholtz 1999). This chapter briefly introduces these aspects with particular emphasis on specific characters of noble crayfish.

Life cycle and reproduction

Mating of noble crayfish takes place in autumn and is triggered by decreasing temperature and day length (Westin and Gydemo 1986) with temperature of major importance (Reynolds et al. 1992). In wild populations mating can be observed in between October and December depending on latitude (Taugbøl and Skurdal 1990; Skurdal and Taugbøl 1994; Reynolds 2002). The development of well-developed glair glands shows sexual maturity of female noble crayfish (Taugbøl and Skurdal 1989) while males gonopods turn white and begin to swell during the breeding period (Skurdal and Taugbøl 2002). While immature eggs are present in the ovaries of adult female noble crayfish all year round, maturation of eggs starts from July on (Tab. 1).

Noble crayfish live singly during the year and mating period is characterised by an increase in activity of both sexes also during daytime. The mating itself is initiated by the male as he grasps the female with his chelae and turns her on the back to deposit a spermatophore with his gonopods near the females gonopores and sometimes on her telson (Dröscher 1906). Females of noble crayfish do not store sperm and spawning takes place a few days to weeks after mating. Female egg-extrusion in crayfish is regulated by hormonal and abiotic cues and takes place also in the absence of males (Woodlock and Reynolds 1988). Shortly before egg-extrusion, the female

retracts the pleon forming a closed chamber for the eggs. A slimy secretion is released from the glair glands that are situated at the ventro-lateral margins of the abdomen. Eggs coming out of the oviduct mix with that secretion, the spermatophore is diluted and the spermatozoa are released. The female continuously moves the pleopodal legs during spawning and thereby, the eggs become attached (Skurdal and Taugbøl 2002).

Table 1: Yearly life-cycle of female *Astacus astacus* in a lake on Gotland (modified from Ackefors 1999). When eggs mature in August, new eggs are produced by the gonads that develop the following year. The period for moulting and subsequent somatic growth for reproducing females is only three to four months while females that loose or are relieved from their eggs in winter or early spring will moult twice such as males.

Month		**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**
Ovarian eggs	white												
	yellow												
	brown												
Pleopodal eggs													
Hatchlings													
Moult													
Glair glands													
Mating													
Surface temperature °C		**0**	**0**	**2**	**5**	**10**	**15**	**22**	**24**	**15**	**10**	**5**	**0**

The number of pleopodal eggs is frequently between 50 and 65 % of ovarian egg number and both are highly variable between individuals within a population (Skurdal and Qvenild 1986). In general, larger females produce more ovarian eggs and are as well capable of carrying more pleopodal eggs (Skurdal and Qvenild 1986), but very large females, however, frequently carry only very small egg numbers (Hager 1996; own unpublished data). Eggs of noble crayfish are relatively large (2.8 to 3.1 mm diameter) and egg size is not correlated with female size (Skurdal and Taugbøl 1994), although pleopodal eggs of very small females are an exception and are frequently much smaller (personal observation). Females usually carry between 150 and 200 pleopodal eggs but up to 360 have been observed (Skurdal and Taugbøl 2002; own unpublished data).

During the period of egg incubation, females are inactive in their shelters and show thorough brooding behaviour. They supply well oxygenated water by fanning of the pleopods,

they clean the egg batches and remove dead eggs (Reynolds 2002). Under natural conditions, juvenile noble crayfish hatch between the beginning of June and mid-July depending on ambient water temperature after approximately 1900 degree days (Reynolds 2002) although the incubation period and the number of degree days until hatching is reduced at elevated temperature (Hessen et al. 1987). All astacid crayfish hatch from the eggs in dekapodit stage (Anger 2001) and become independent from their mother after the first moult to stage II in Astacidae or after the second moult in Parastacoidea and Cambaridae (Reynolds 2002). The absence of a pelagic juvenile stage is characteristic for all crayfish species and considered an adaptation to their freshwater habitats (Scholtz 1999).

Sexual maturity is reached at approximately 60 mm (males) and 70 mm (females) total length and it takes three to five years for noble crayfish to reach sexual maturity in natural waters (Skurdal and Taugbøl 1994; Ackefors 1999). In Northern Europe, females spawn in alternate years (Skurdal and Taugbøl 1994) because the period between release of juveniles and the subsequent spawning season is too short to reach sexual maturity in northern climate (Tab. 1). In Central Europe, however, females are capable of reproducing every year due to the earlier release of juveniles and later start of the breeding season (Reynolds 2002).

Growth and moulting

"*The heavy exoskeleton and its moulting cycle dominate the life of these animals*" (Reynolds 2002).

There are two distinct growth types in freshwater crayfish that differ between warm-water and cold-water species. While continues growth showing r-selected characteristics is typical in subtropical environments, seasonal growth occurs in temperate, often K-selected species like noble crayfish (Reynolds 2002). All European astacid crayfish show a seasonal growth pattern with significant somatic growth only occurring during summer months. Therefore, the terms "summerlings" and "two/three/etc. summer old crayfish" are frequently used to describe growth of a certain age group rather than referring to the age in years.

Crayfish just like any other crustaceans have to moult to grow and any increase in size is only possible immediately after moulting (Green 1961). During the period between two moults when the exoskeleton is fully hardened (intermoult period), crayfish feed, build up energy reserves and reproduce. During the moulting process, however, the exoskeleton gets soft and

crayfish are highly vulnerable to environmental factors and predation (Romano and Zeng 2017). Hormonal control of moulting primarily involves the X-organ-sinus gland system located in the eyestalks (Burghause 1975) and the paired Y-organs that are located in the epidermis posterior-laterally to the oesophagus (Andrew et al. 1978). The moulting hormone ecdysone is released from the Y-organ while moult inhibiting hormone (MIH) is released from the X-organ and during intermoult, release of ecdysone is supressed by MIH (Vogt 2002). The moulting process involves several steps controlled by physiological changes that are basically regulated by ecdysone (Reynolds 2002). Preparation for moulting starts with apolysis of the old cuticule and the formation of the epicuticule (van Herp and Bellon-Humbert 1978) and the resorption of calcium from the inner layers of the exoskeleton. Most of the calcium is lost to the ambient water through the gills but approximately 10-20 % are stored in the gastroliths (Greenaway 1985). While the pre-moult period in noble crayfish extends over several days, shedding of the exoskeleton itself (ecdysis) takes only a few minutes (Wolf 1976). A complete moult comprises the shedding of the cuticle from all exoskeletal parts including the surface of the eyes and the ectodermal linings of fore- and hindguts (Reynolds 2002). During early post-moult, crayfish take up water to the haemolymph primarily through the hindgut to increase size (Vogt 2002) and Na^+, Mg^{2+} and Cl^- through the gills (Freire et al. 2007) to restore ionic equilibrium (Zare and Greenaway 1998). Furthermore, the process of calcification requires intense uptake of calcium from the water which is highly demanding for hyper-regulating freshwater crayfish (Wheatly and Gannon 1995) as ion uptake is an active process (Vogt 2002; Freire et al. 2007). Suitable water parameters are crucial for successful moulting and post-moult exoskeleton mineralisation and extreme pH (Jay and Holdich 1977; Haddaway et al. 2013; Beaune et al. 2018), low alkalinity (Greenaway 1974) and low ambient calcium concentrations (Wheatley and Ayers 1995; Rukke 2002; Cairns and Yan 2009) therefore may limit distribution of crayfish and cause increased mortality or reduced growth in culture.

Size and weight gain of crayfish within a growing season are important parameters likewise for a viable culture and for population dynamics in natural water bodies. Size increment per moult and moulting frequency or length of intermoult period, respectively, determine growth in crayfish. Consequently, environmental factors determining growth are numerous and frequently interactive. Given suitable water chemistry for moulting and post-moult exoskeleton mineralization, temperature, dissolved oxygen and food availability are recognised as most significant for crayfish to grow (Skurdal and Taugbøl 2002). After the first summer, noble crayfish usually do not exceed 1.5 g total weight and 30 mm total length (Keller 1988; Westman et al. 1993; Tulonen et al. 1995; Skurdal and Taugbøl 2002). Accelerated incubation may, however,

extend the period available for growth thereby facilitating a higher number of moults and consequently, higher individual weight (4-5 cm total length and 6-7 g total weight) at the end of the growing season (Jeske 2007; Lehmann et al. 2013). For juvenile noble crayfish with high moulting frequency, temperature is particularly relevant as it has great influence on the total number of moults (Kouba et al. 2010). Moulting is not observed at water temperatures below 10 °C (Henttonen et al. 1993) and for noble crayfish, optimum temperature for moulting and subsequent growth ranges from 16 – 24 °C (Jussila 1997; Füreder et al. 2006; Füreder 2016). Consecutive intermoult periods extend with every moult (Ackefors et al. 1995, Kouba 2010) and under experimental conditions, juvenile noble crayfish moult six (Ackefors et al. 1995) to eight (Wesenberg-Lund 1939) times in the first summer. Adult males, however, moult twice each year while females that reproduce only moult once while, as they will carry their eggs until mid-June and are inactive during this period (Ackefors 1999; Tab. 1). Consequently, size and weight gain of reproducing females within a growing season is smaller than in mature males.

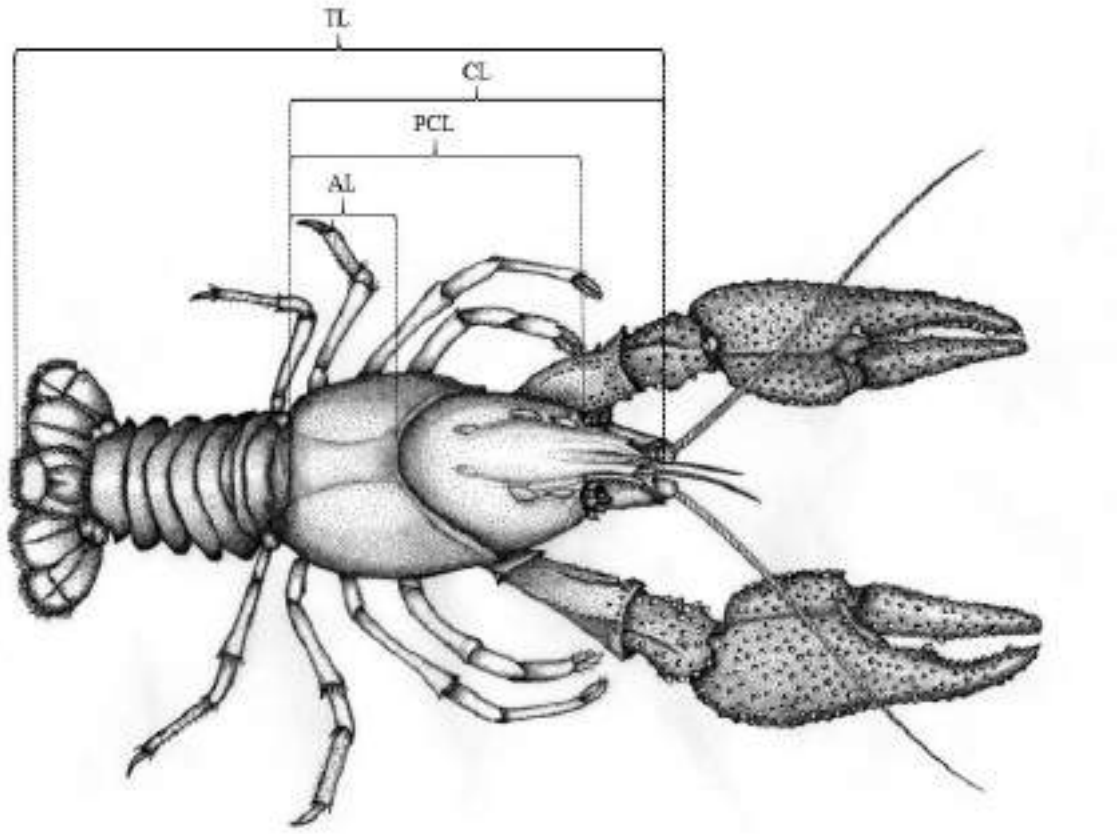

Figure 1: Drawing of an adult male noble crayfish showing frequently used parameters for measuring size (i.e. length). TL = total length; CL = carapace length; PCL = postorbital carapace length; AL = areola length. Modified from S. Geisler.

To assess of growth in crayfish different morphometric and physiological parameters are applied. Size (i.e. length) is typically reported as total length from the tip of the carapace to the end of the telson (Fig. 1). Furthermore, carapace length is commonly reported instead of or in addition to total length. Carapace length can be more accurately determined than total length, as measuring the latter requires stretching the pleon. Fitzpatrick (1977) evaluated alternate techniques to measure carapace length and concluded that, despite the importance of areola length for taxonomy, standard carapace length from the tip of the rostrum to the end of the carapace is most suitable for reporting the animals' size. The relationship between total length and standard carapace length is consistently between 1.9 to 2.0 in noble crayfish (unpublished data) and for the purpose of culture studies, both parameters are suitable. Weight gain is most important in aquaculture as it determines yield. Consequently, initial and final weight and indices based on these parameters are usually reported. Comparison of different studies is, however, difficult if solely based on wet weight, as this parameter neglects the time required to grow to the reported weight. Therefore, Evans and Jussila (1997) assessed different growth indices that account for time (absolute growth rate: mg per day; relative growth rate: percent increase wet weight per day) and recommended the use of specific growth rate ($SGR = \ln(W_f) - \ln(W_i) / t$, where W_i is the initial weight, W_f is the final weight and t is the duration of the study in days). They argue that SGR is based on an exponential growth trend which corresponds to the observed growth pattern in cultured juvenile and early adult crayfish (Evans and Jussila 1997). In addition to these morphometric measurements, nucleic acid concentrations, in particular RNA, have been used to assess physiological condition of crayfish, thereby accounting for the animals' discontinuous growth (Edsman et al. 1994; Grimm et al. 2015; Roessler et al. 2020) or to assess physiological condition in field studies (Olsson et al. 2008).

All astacid species show similar sexual dimorphism in adults. While growth is isometric in juveniles and females, the level of allometry increases at pre-puperty moults in males, in particular, as their chelae grow larger (Rhodes and Holdich 1979). Mature males and females further differ in the width of the abdomen which is wider in females than in males while males generally reach a higher final weight. This sexual dimorphism has implications for culture as males have more meat in their chelae and females have more tail meat (Rhodes and Holdich 1979; Harlioğlu and Holdich 2001). Maximum size of noble crayfish usually does not exceed 15 cm total length, although lager males up to 20 cm and 350 g wet weight have been reported (Hager 1996; Kouba et al. 2015). Therefore, noble crayfish represent the largest mobile invertebrates in European freshwater ecosystems and are able to shape their environment physically and to influence biotic interactions by their foraging behaviour (Nyström 1999).

Circadian locomotor activity and light perception

"*Tagsüber hält sich der Krebs meistens in Verstecken verborgen in seinen selbstgegrabenen Höhlen am Ufer oder unter Steinen zwischen Wurzelgestrüpp der Uferbäume*" (Dröscher 1906).

Crayfish generally exhibit a diurnal activity pattern with activity concentrated during nocturnal hours (Gherardi 2002; Fanjul-Moles and Prieto-Sagredo 2003; Styrishave et al. 2007) and peaks of activity around onset of dark and light phase are observed in astacid crayfish (Cukerzis 1988; Page and Larimer 1972; Fuentes-Pardo et al. 2003; Miranda-Anaya 2004). In astacid crayfish, this pattern is particularly apparent during the growing season and supressed during mating and in the winter (Barbaresi and Gherardi 2001). Light is the primary external stimulus that induces this bimodal locomotor activity and it has been demonstrated that both the onset of the light and dark phase is often anticipated (Aréchiga et al. 1993; Fernández de Miguel and Aréchiga 1994; Sullivan et al. 2009). In accordance with Aschoff`s rule (Aschoff 1960) the locomotor activity pattern changes to a free running period of approximately 24 hours if the external stimulus light is omitted (Page and Larimer 1972; Häberle 2013).

Crayfish possess three distinct photoreceptor systems, the compound eyes located on the eyestalks, a caudal photoreceptor in the terminal abdominal ganglion and extraretinal brain photoreceptors in the supraesophageal ganglion (Sullivan et al. 2009). The compound eyes are the visual organs of crayfish and consist of 635 (juvenile *A. astacus*) to 3050 (adult *A. astacus*) ommatidia. They are superposition eyes typical for arthropods being active in dim light. However, movements of the screening pigments ease adaptation to strong light as well (Vogt 2002). The caudal photoreceptor is a nonvisual photosensitive neuron that shows spontaneous activity to pulses of light. Its function is yet not fully understood but it apparently participates in the entrainment of circadian locomotor rhythms (Rodríguez-Sosa et al. 2012; Sánchez-Hernández et al. 2018). The photosensitive pigments of the brain photoreceptor primarily attain light through the translucent eyestalk membrane (Sandeman et al. 1990). Their function as circadian photoreceptors for the entrainment of circadian rhythms is underlined by the projection to two small neuropils where they terminate among fibres expressing the neuropeptide pigment-dispersing hormone that is a signalling molecule in arthropod circadian systems (Sullivan et al. 2009).

An ontogenetic shift in the spectral sensitivity from blue to red light has been demonstrated for the compound eyes (Fanjul-Moles and Fuentes-Pardo 1988; Fanjul-Moles et al. 1992) and there is evidence that the entrainment of the circadian rhythm is not distinct in very

young (< 3 weeks) animals (Fanjul-Moles et al. 1992). An ontogenetic shift in spectral sensitivity has, however, not yet been confirmed for extraretinal photoreceptors involved in the entrainment of circadian rhythms. However, extraretinal photoreceptors seem to be more sensitive to green and blue than to red light (Sánchez-Hernández et al. 2018) caused by the presence of the short (440 nm) and long (530 nm) wavelength sensitive opsins in the caudal photoreceptor and the ganglia of the nerve cod (Kingston and Cronin 2015).

Food and feeding

"*Few things in the way of food are amiss to the crayfish; living or dead, fresh or carrion, animal or vegetable, it is all one*" (Huxley 1880).

Crayfish are important omnivorous consumers in aquatic food webs and may dominate the aquatic biomass in lakes and streams (Nyström 1999; 2002). Crayfish have the ability to shape the physical structure of their environment, significantly influence biotic interactions and are therefore considered keystone species or ecosystem engineers (Momot 1995; Dorn and Wojdak 2004). Most important impacts include top-down effects on aquatic macrophytes and periphyton (Flint and Goldman 1975; Dorn and Wojdak 2004; Klose and Cooper 2012), fish (Peay et al. 2009) and benthic macroinvertebrates (Dorn and Wojdak 2004; Klose and Cooper 2012). Furthermore, direct consumption of leaf litter (Hessen and Skurdal 1986; Huryn and Wallace 1987), zooplankton (Keller 1988; Pullwitt 2010), periphyton (Hessen and Skurdal 1986), macroalgae (Kholodkvitch et al. 2005), macrophytes (Nyström and Strand 2003), macroinvertebrates (Hessen and Skurdal 1986; Momot 1995; Stelzer and Lamberti 1999) and fish (Reynolds 2011) has been thoroughly documented.

The extend of dietary contribution of a given food source may vary according to species (Johnston et al. 2011), age (Westman et al. 1986; France 1996), habitat (Johnston et al. 2011) or season (Beatty 2006). Grazing represents a major foraging strategy for omnivorous crayfish (Flint and Goldman 1975; Evans-White and Lamberti 2005) and periphyton is common in the natural diet of noble crayfish (Hessen and Skurdal 1986; Westman et al. 1986) while Olsson et al. (2008) found, that the dominant food source contributed most to the animals' diet and growth. To current knowledge, however, animal food is of greater importance for juveniles than for adults (Abrahamsson 1966; Mason 1975; Nyström 2002) and consistent with this observation, juveniles can be reared with reasonable success on a commercial diet containing approximately 50 % crude

protein (Carral et al. 2011; Fuertes et al. 2013a; 2013b; 2014) or if animal food supplements a high protein diet (e.g. González et al. 2008).

A powerful tool to study the contribution of a given food source to an animals' diet is the application of stable isotope analysis (DeNiro and Epstein 1978; Schroeder 1983). This method is frequently used to assess the trophic position and diet of omnivorous crayfish (e.g. Stenroth et al. 2006; Olsson et al. 2008; Joyce and Pirozzi 2016; Marufu et al. 2018). Abdominal muscle tissue is most frequently analysed in studies with crayfish (e.g. Stenroth et al. 2006) and by far most common elements are carbon and nitrogen that both have two stable isotopes (^{12}C, ^{13}C and ^{14}N, ^{15}N). Ingested food items have a distinct ratio of heavy to light isotope that define their isotopic signature. Isotopic signatures are reported in delta (δ) units in parts per thousand (‰) relative to a standard (Vienna Pee Dee Belemnite for C; atmospheric nitrogen for N) and calculated as follows:

$$\delta X = (R_{sample} / R_{standard} - 1) \times 1000,$$

where X = ^{13}C or ^{15}N and R = ^{13}C / ^{12}C or ^{15}N / ^{14}N, respectively. Animals that feed on a given food source reflect the isotopic signature of the respective food source in their tissues (Fry 2007). Due to metabolic processes, however, there is an important change in the isotopic signature between diet and consumer (Tieszen et al. 1983; Caut et al. 2008a; 2009; Carolan et al. 2012). This change is termed isotopic discrimination (or fractionation) and the difference between the isotopic signature of died and consumer consequently referred to as fractionation factor Δ. Fractionation for carbon typically is around 0.4 ‰ while fractionation for nitrogen is around 3.4 ‰ from one trophic level to the next in aquatic food webs (Post 2002).

If trophic discrimination for a given food source is known or can be estimated (Caut et al. 2009), it is possible to calculate the contribution of a dietary source to a consumer's whole body or tissue and recent Bayesian mixing models even allow estimating the dietary contribution several food sources to a consumers' diet (Moore and Semmens 2008; Phillips 2012; Stock et al. 2018). This approach has recently received attention in crayfish culture to disentangle the importance of naturally occurring food items and commercial feeds (Duffy et al. 2011; Viau et al. 2012; Joyce and Pirozzi 2016; Jin et al. 2019a). The outcome of the mixing model is, however, highly sensitive to variations in Δ (Caut et al. 2008b; Bond and Diamond 2011). Consequently, for several of crayfish species Δ values have been experimentally determined for $\delta^{13}C$ and $\delta^{15}N$ using different diets (e.g. Glon et al. 2016; Laurenz 2014) and tissues (e.g. Mazumder et al. 2018).

Culture of freshwater crayfish

"*The rearing methods used to culture Astacus vary considerably, but the technology is generally very simple*" (Skurdal and Taugbøl 2002)

Crayfish culture, like culture of other (aquatic) animals, aims at producing crayfish at relatively high densities and relatively low costs (Keller 1988). Today, due to the severe loss in biodiversity of freshwater crayfish in Europe, their culture may provide various direct and indirect values for society in addition to the production of high quality food (Gherardi 2011).

Global situation of freshwater crayfish culture

Crayfish culture is practiced virtually around the globe in all regions where crayfish naturally occur or have been introduced and encompasses various species (Policar and Kozák 2015). In 2018, crayfish production accounted for 1.5 percent of the total global aquaculture production of 114.5 million tonnes and crayfish are ranked 13 of 50 cultured species groups (FAO 2020). With a 43 percent annual production increase from 2017 to 2018 crayfish constitute, however, the fastest growing of all FAO species groups. Notably, the value of produced crayfish makes up 5.49 percent of world aquaculture farming value which ranks crayfish 4th, following cyprinids, marine shrimps and prawns and salmonids (FAO 2020).

By far the most important cultured crayfish species is the red swamp crayfish *Procambarus clarkii* (Girard, 1852). This member of Cambaridae accounts for 99.98 percent of total global crayfish production and is mainly produced in China (95,74 %) and the United States of America (4,25 %) (FAO 2020). Production is primarily based on semi-intensive culture in earthen ponds, rice-fields and swamps from where *P. clarkii* is harvested for consumption purpose (Huner 2002, Policar and Kozák 2015). Among the Cambaridae are only a few other species that are of interest in aquaculture. While *Procambarus acutus* (Girard, 1852) is cultured in North America frequently together with *P. clarkii* for consumption (Huner 2002), *Orconectes immunis* (Hagen, 1870) is produced for the fishing bait industry and commercial implementation of other *Orconectes* species has so far failed to establish (Hamr 2002).

The southern hemisphere family Parastacidae includes three warm-water crayfish species of commercial importance, namely smooth marron (*Cherax cainii* Austin and Ryan, 2002; formerly *Cherax tenuimanis* Smith, 1912), yabby (*Cherax destructor* Clark, 1936) and red claw

crayfish (*Cherax quadricarinatus* von Martens, 1868) (FAO 2020b). Those are primarily produced in tropical and subtropical regions of Australia, Asia, South and Central America (Merrick and Lambert 1991; Garca de Yta 2009; 2016) and intensification of their culture has recently received considerable attention (Garca de Yta 2009; 2016; Jones and Valverde 2020; Rigg et al. 2020). Even though their life cycle differs significantly from cold-water astacid crayfish in temperate regions (Skurdal and Taugbøl 2002; Lewis 2002; Lawrence and Jones 2002), artificial incubation of eggs (e.g. Reynolds et al 1992; Jones and Valverde 2020) and the implementation of a nursery phase (Geddes et al. 1995; Kouba 2011) are, among others, approaches to an intensified culture that show similarities in cultured members of the Parastacidae and Astacidae.

Within the Astacidae, narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823), signal crayfish *Pacifastacus leniusculus* (Dana, 1852), *Austropotamobius pallipes* (Lereboullet, 1858) and noble crayfish *A. astacus* (L. 1758) are cultured, predominantly in Europe (Huner 1994; Skurdal and Taugbøl 2002; Lewis 2002). While production of *A. pallipes* basically serves species conservation due the species' rarity and European legislation (European Commission 1992), production of narrow-clawed crayfish primarily takes place for human consumption (Policar and Kozák 2015). Aquaculture of *P. leniusculus* and *A. astacus* delivers both crayfish for human consumption and for restocking (Ackefors and Lindqvist 1994; Keller 2009) but commercial use and culture of signal crayfish in the European Union have, however, recently been restricted to reduce the spread of this non-indigenous crayfish in the European Union (European Commission 2014).

Development of cold-water astacid crayfish culture in Europe

Until the middle of the 19th century, there was no need to culture crayfish in Europe – they were abundant in almost any kind of freshwater habitat and were captured from wild populations (Keller 2009). After the devastating spread of the crayfish plague *Aphanomyces astaci* (Schikora, 1906) in 1860 caused an area-wide population collapse, first attempts to culture crayfish were made to sustain the valuable crayfish fisheries (Dröscher 1906; Schikora 1916). Until the 1970s, however, these were restricted to the production of juvenile crayfish for the restocking in open waters were natural populations had been depleted (Cukerzis 1973; Strempel 1973). In Scandinavia, where crayfish are of great cultural importance (Swahn 2004), thorough evaluation of noble crayfish culture was conducted during the 1980s and 1990s (Gydemo 1995). Several studies revealed the suitability of noble crayfish for a semi-intensive production in earthen ponds (e.g. Pursianen et al. 1983; Gydemo and Westin 1988; Keller and Keller 1995) while year-round

intensive indoor culture was found not to be profitable (Gydemo 1995; Jussila 1997), in particular due to the space requirements of crayfish after the first summer and until they reach table-size after three to four summers. Beside culture of noble crayfish, the potential of signal crayfish for culture purpose was evaluated (Mason 1978; Tulonen et al. 1995). As signal crayfish show higher growth rates in juveniles (Tulonen et al. 1995) they were sometimes considered superior to noble crayfish for culture (Gydemo 1995).

Control over hatching and acquisition of early juveniles was a major issue already in the beginning of commercial crayfish culture in Europe. It was recognised that crayfish are most effectively cultured in ponds that are stocked crayfish of the same age (Keller 1988; Keller and Keller 1995). To achieve this, two approaches were established as an important step to an intensification of culture. In the first case, egg-carrying females are removed from the ponds shortly before the juvenile crayfish hatch from the eggs in late May or at the beginning of June. Females are subsequently transferred to separate holding tanks, frequently equipped with artificial shelter and a perforated double bottom, where the juveniles hatch. After they have become independent from their mother after the first moult, juvenile crayfish are stocked into earthen ponds (e.g. Pursianen et al. 1983; Keller 1988). A second approach is the reproduction (i.e. mating, spawning and hatching) under controlled indoor conditions (Hessen et al. 1986; Taugbøl and Skurdal 1989; 1990). In this case, sexually mature crayfish are obtained from wild or culture stocks in autumn and transferred to recirculating or flow-through aquaculture systems at preferably low densities (Taugbøl and Skurdal 1990) that make it unnecessary to separate males after mating (Carral et al. 2000). Optimum density for reproduction of noble crayfish in captivity was found to be 1 male per 3 females (Taugbøl and Skurdal 1989), a ratio that was later basically confirmed for signal (Celada et al. 2005) and white-clawed crayfish (Sáez-Royuela et al. 2005), although males are capable to mate with 15 to 30 females (Policar and Kozák 2015).

Today, culture of noble crayfish commonly still is year-round culture in earthen ponds that are managed either extensive or semi-intensive (Ackefors 2000; Keller 2009; Policar and Kozák 2015). Surface area of crayfish ponds rarely exceeds 1 ha (Ackefors and Lindqvist 1994) and ponds are frequently narrow to enhance shoreline/area ratio as crayfish shelter are commonly placed along the shoreline in shallow water. The animals predominantly feed on naturally occurring food items and wheat or commercial aquaculture feeds are additionally supplied (Keller 2009). It is difficult to state the total aquaculture production of noble crayfish in Europe as statistics from various sources differ considerably and frequently do not differentiate between harvest from natural stocks and aquaculture production (Skurdal and Taugbøl 2002, Policar and

Kozák 2015). Yearly yields from semi-intensive pond culture range from 100 kg/ha in Bavaria (Keller and Keller 1995) to 60 – 430 kg/ha in Sweden (Ackefors 1997; Ackefors 2000) and may be up to 600 kg/ha in favourable years in northern Germany (Ackefors 2000; Jeske, personal communication).

Current development and constraints in culture of cold-water astacid crayfish

The culture of crayfish for consumption and stocking has a long tradition in Europe (Swahn 2004) and has gained importance since the decline of natural stocks of indigenous noble crayfish (Dröscher 1906). There are, however, several biological constraints to productivity in cold-water-adapted astacid crayfish and overcoming them is critical for economically successful aquaculture. Longevity, slow and seasonal growth, a short growing season and low fecundity are challenging, general life cycle characteristics (Huner and Lindqvist 1991) and under culture conditions, density dependent cannibalism, reduced survival and retarded growth are inherent (Keller 1988; Mazlum 2007; Romano and Zeng 2017).

Several attempts were made to enhance productivity of cold-water astacid crayfish by establishing intensive indoor-culture in recirculating aquaculture systems (RAS), thereby facilitating year-round growth at optimum water temperature thereby solving the “seasonal growth” problem (e.g. Jussila 1997; Franke et al. 2013; Seemann et al. 2015). Those studies have enhanced our knowledge on physiological and behavioural response of crayfish to intensive culture conditions and aided the understanding of moulting cycles and nutritional requirements. It can, however, be stated that year-round production in RAS even though it is biologically feasibility, has never been established anywhere in the world for any species due to the space demands table-size crayfish, their cannibalistic behaviour and the lack of suitable grow out feeds (Huner 1994; Huner 2002; Lewis 2002; Skurdal and Taugbøl 2002; Kozák et al 2015; Garca de Yta 2016. Nevertheless, there are several other promising approaches to the intensification of culture that also address the above mentioned problems. In particular, accelerated egg development and intensification of juvenile rearing are auspicious to enhance efficiency of astacid culture.

Accelerated incubation of crayfish eggs

A popular approach of astacid culture has been to shorten the period from spawning to hatching by an accelerated incubation of eggs (e.g. Reichenbach 1886; Mason 1977b; Pérez et al. 1998a; Carral et al. 2003) to increase the animals' growth period in the first year (Huner and Lindqvist

1991). Water temperature is crucial for embryonic development of crayfish eggs as it directly influences their embryonic development and time to hatching (Celada et al. 1988; Pérez et al. 1998a; Celada et al. 2001; Policar et al. 2004; González et al. 2009a; Jin et al. 2019b). Cukerzis et al. (1978) and later Taugbøl and Skurdal (1995) concluded from the long incubation period that *Astacus astacus* requires a period of cold ambient water temperature to successfully complete embryonic development. Information on the duration and optimal time point of the cold period for successful embryonic development for astacid crayfish is, however, inconsistent. Observational and experimental studies confirmed that a cold period improves survival in signal crayfish *Pacifastacus leniusculus* (Celada et al. 1988; Carral et al. 1992). Rhodes (1981) found similar beneficial effects of a cold period for *Austropotamobius pallipes* whereas Pérez (1998a) concluded that a cold period did not positively affect survival in the same species. For *A. astacus*, however, information on the optimal temperature regime and cold period for accelerated embryonic development is lacking.

For artificial incubation of crayfish eggs (i.e. incubation of eggs separately from the female), professional incubation devices have been developed (Järvenpää and Ilmarinen 1990; Jones and Valverde 2020) and it is widely accepted that artificial incubation is preferable for experimental studies as they allow decoupling of maternal and embryonic responses to experimental variation. For culture purposes, space and energy requirements are significantly reduced, and survival rates are generally higher in artificial than in maternal incubation (Pérez et al. 1999). Egg losses during artificial incubation are frequently caused by fungal infections as maternal care of the eggs is missing. Therefore, attempts to improve incubation efficiency during artificial incubation by daily removal of dead eggs (Policar et al. 2006), treatment of process water with ultraviolet light (Kouba et al. 2013), regular egg bath wit antifungal treatments (Policar et al. 2006; González et al. 2013; Seemann et al. 2014) have been evaluated and are generally applicable for astacid culture.

High mortality usually occurs shortly before and at hatching and is further enhanced in unsuitable incubation regimes due to an exhaustion of metabolic reserves (Reynolds 2002; García-Guerrero et al. 2003). Furthermore, mortality of earlier hatched and already moulted stage II juveniles on their late-hatched conspecifics is a major issue in artificial incubation (Melendre et al. 2007; Shun et al. 2020).

Overall, species specific dormancy mechanisms are still poorly understood and important for large scale application of accelerated incubation. To overcome these limitations, knowledge on the duration of a cold period for successful embryonic development is important as

it may significantly improve survival and positively affect stage II juvenile fitness. Only if early hatched animals perform similar to (Sáez-Royuela et al. 1995) or even better than their late hatched conspecifics (González et al. 2009a) the advantage of a longer growth period can be converted into higher biomass at the end of the growing season.

Assessment of physiological condition

The cultivation of aquatic animals under controlled conditions requires not only the development of specific culture techniques but also depends on the appropriate assessment of the animals' physiological condition. Research on culture intensification in crayfish apply somatic growth rate described as an increase in wet weight or body length. However, high individual variation within an age group along with intermittent growth result in a restriction of temporal resolution and loss of accuracy. Recently, there is growing interest in the development of formulated feeds for crayfish (Garca de Yta 2009, González et al. 2012; Fuertes et al. 2013a; 2013b; Hua et al. 2014) and accurate assessment of growth is crucial for its success. If overall growth capacity is of major interest, the assessment of RNA content is a promising approach as protein synthesis also occurs during intermoult, whereas size and weight do not change much during this period as water content of tissues is replaced by proteins during somatic growth (Edsman 1994). Protein synthesis, however, depends on ribosomal activity and the synthesis of RNA and as the DNA content in cells is fairly constant, the ratio between RNA and DNA reflects the rate of protein synthesis. Furthermore, RNA/DNA ratio can be expected to indicate the suitability of different feeds more quickly than weight or length related parameters in crayfish.

Nursery of noble crayfish

Under natural conditions, juvenile crayfish hatch between the beginning of June and July. If early hatched juveniles are produced for culture by an accelerated incubation of crayfish eggs, they need to be reared in closed nursery systems until ambient conditions are suitable for pond stocking (Fig. 2). On the one hand, the prolonged growth period favours higher final weight after the first growing season. On the other hand, if advanced juveniles are stocked into ponds at the same time when they wold have hatched without accelerated incubation, their increased size makes them less vulnerable to predation by predatory aquatic insect larvae.

During intensive nursery, however, mortality and reduced growth rates caused by cannibalism and agnostic interactions pose the greatest challenge freshwater crayfish production (González et

al. 2009b; González et al. 2011; Romano and Zeng 2017). To minimise losses due to agnostic interactions, shelter availability (Mason 1978; Kozak et al. 2001; Baird et al. 2006), food quality (Saez-Royuela et al. 2007; González et al. 2008; Saoud et al. 2012), density (Keller 1988; Morissy et al. 1995; Mazlum 2007; Ramalho et al. 2008; Torno 2011) and lighting conditions (Mason 1978; González et al. 2011, Franke and Hörstgen-Schwarck2015) are important. Among these, the identification of optimal lighting conditions (intensity, spectral composition) is a promising approach as lighting may influence behavioural patterns of the animals (Arechiga and Rodríguez-Sosa 1997; Farca Luna et al. 2009) thereby reducing agnostic interactions and cannibalism (Farca Luna et al. 2009; Franke and Hörstgen-Schwark 2015).

In addition, lighting can strongly affect periphyton growth in aquaculture rearing tanks (Wasielesky et al. 2012). Grazing represents an important foraging strategy for omnivorous crayfish (Flint and Goldman 1975; Evans-White and Lamberti 2005) and periphyton is common in the natural diet of noble crayfish (Hessen and Skurdal 1986; Westman et al. 1986). In semi-intensive aquaculture of crayfish, naturally occurring food items significantly contribute to growth even if formulated feed is provided (Henttonen et al. 1993; Duffy et al. 2011; Viau et al. 2012; Jin et al. 2019a) and highest growth and survival rates are frequently obtained in more natural environment (Pursiainen et al. 1983) or if natural food is supplied in addition to formulated feeds (Henttonen et al. 1993; Sáez-Royuela et al. 2007; González et al. 2008; 2011; Viau et al. 2012). Therefore, knowledge on the interactive effects of different lighting conditions on crayfish performance and periphyton growth is important for the implementation of a nursery phase for noble crayfish.

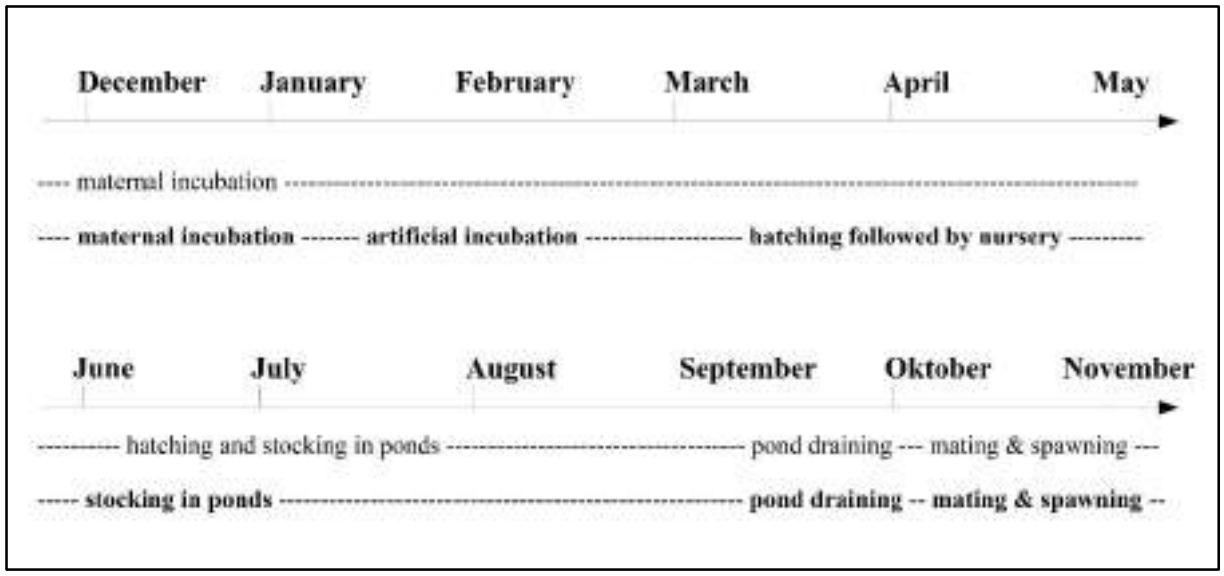

Figure 2: Generalised production cycle of noble crayfish in northern Germany. The approach to culture intensification with accelerated egg-incubation and nursery is written in bold letters. The growth period of juvenile crayfish is extended by approximately three months in the first growing season

THESIS OUTLINE

The main part of my thesis is structured in three chapters each in form of a manuscript containing an abstract, introduction, materials and methods, results and discussion. The first manuscript on the significance of a cold period for embryonic development of noble crayfish is published in "*Invertebrate Reproduction and Development*". The second manuscript on the suitability of RNA/DNA ratio as a performance parameter for juvenile noble crayfish is published in "*Aquaculture Research*". The third manuscript on the effects of stocking time and light sources during nursery of noble crayfish is prepared for submission to "*Journal of Applied Aquaculture*".

I developed the idea for the thesis within a project on intensification of noble crayfish culture funded by a European Fisheries Fund Grant to Prof. Dr. Heinz Brendelberger under Grant SH-363E.35. The project was conducted in close cooperation between the Limnology Group at Kiel University and Helmut Jeske, Oeversee Crayfish Farm, Oeversee, Germany. I developed the concept for the thesis under scientific advice from Prof. Dr. Heinz Brendelberger and with practical advice from Helmut Jeske. All experiments were performed at the facilities of Kiel University (Chapter I, Chapter II) or at Oeversee Crayfish Farm (Chapter III). Laboratory analysis were performed at Kiel University (Chapter I, Chapter II, Chapter III) and at GEOMAR Helmholtz-Zentrum für Ozeanforschung Kiel (Chapter II).

The aim of my thesis is to promote an ecological and economical feasible intensification of noble crayfish culture through improved hatchery and nursery accompanied by an accurate assessment of the animals' physiological condition. The main approach is the shortening of embryonic development followed by a nursery phase until ambient conditions are suitable for pond stocking. On the one hand, this facilitates a shortened production period of table size crayfish and an improved rearing of juvenile crayfish from endangered populations for restocking of natural waterbodies. On the other hand, my findings contribute to our general knowledge on noble crayfish biology and support further studies on the development of suitable rearing conditions and the development of adequate feeds.

Chapter I

Embryonic development of noble crayfish is characterised by a long dormant period during winter months. In astacid crayfish, this period can be significantly shortened by elevated temperatures to obtain early hatched juveniles. This is crucial for the implementation of a nursery phase in culture

of noble crayfish to enhance the animals' growth period in the first year. If dormancy of noble crayfish represents a form of true diapause, its shortening may be achieved only at the expense of reduced survival and juvenile fitness. However, only if early hatched juveniles perform similar or better than their late hatched conspecifics, the extended growth period can be converted into higher biomass at the end of the growing season. Therefore, I studied the effect of different extended cold periods during artificial incubation of early stripped eggs on embryonic development, incubation efficiency and subsequent juvenile crayfish performance in noble crayfish. My findings support the hypothesis that dormancy in noble crayfish represents true diapause as hatching success and stage II juvenile weight increased with increasing cold period. In addition, the longest cold period synchronised hatching and moulting which has strong implications for culture application as cannibalism of stage II juveniles on eggs and hatchlings frequently causes high mortality. The longest cold period favours production of stage II juveniles in the middle of March which is an optimal time point for the start of a nursery phase.

Chapter II

In the second chapter of my thesis, I studied the suitability of different morphometric and nucleic acid parameters to assess the physiological condition of juvenile noble crayfish as recent research on the intensification of astacid culture has highlighted the need for a valid assessment of the physiological condition. In particular, the discontinuous growth pattern of crayfish and high individual variation within an age group limit the applicability of morphometric growth parameters to accurately assess overall growth. I designed an experiment with different feeding regimes to compare the relationship between somatic growth (carapace length, wet weight, specific growth rate) and nucleic acid based indicators (RNA/DNA ratio, RNA per unit wet weight). Based on RNA/DNA ratios different feeding regimes were detected earlier and RNA/DNA ratio was clearly superior to RNA per unit wet weight, as the latter failed to detect any differences between groups. I could show that RNA/DNA ratio is a suitable parameter in nutritional studies with freshwater crayfish if overall growth is the key variable of interest. Its application may favour the upcoming development of commercial feeds for crayfish of different age.

Chapter III

In the third chapter, I conducted two consecutive on-farm experimental trials to study the effects of stocking time and lighting on the performance of juvenile noble crayfish during an eight-week

nursery phase. Heating of process water during both trials was dependent on ambient conditions and elevated by a greenhouse. Lighting conditions are known to affect growth and survival of juvenile crayfish by influencing agnostic interactions and cannibalism. As lighting favours periphyton growth and this food source is important in the diet of crayfish, I studied the relationships between crayfish performance and periphyton characteristics. I observed different effects of stocking time and lighting on periphyton characteristics and juvenile crayfish performance. Crayfish stocked later grew faster as a result of higher temperatures. Stable isotope analysis revealed differences in the importance of naturally occurring food items for crayfish between light sources. The results illustrate the feasibility of energy efficient nursery phase, show the importance of periphyton as a complementary food source for juvenile crayfish and hint at how its growth may be promoted by novel lighting techniques.

CHAPTER I

EMBRYONIC DEVELOPMENT, ECLOSION AND PERFORMANCE OF JUVENILE NOBLE CRAYFISH *ASTACUS ASTACUS* (L. 1758) IN RESPONSE TO SHORTENED COLD PERIOD

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hatching synchrony; aquaculture; artificial incubation; diapause

Abstract

A suitable temperature regime in artificial incubation of crayfish eggs is crucial for successful application in aquaculture production and conservation projects. We studied the effect of different extended cold periods (CP: 10, 24 and 38 days at 7.5 ± 0.5 °C) during artificial incubation on embryonic development, incubation efficiency and subsequent juvenile crayfish performance in noble crayfish. Best results were obtained at 38-day CP with stage II survival rate of 60.4 ± 7.4 % and stage II wet weight of 38.4 ± 1.9 mg. This regime synchronised embryonic development and equally reduced hatching and moulting period to 7 days with no overlap between both. Survival was high in a subsequent 100-day rearing experiment (10-day CP: 85.0 ± 9.2 %; 24-day CP: 90.0 ± 4.1 %; 38-day CP: 92.5 ± 2.9 %) and juvenile crayfish from 38-day CP grew faster, but these results were not significant. Staggered response of embryonic development to temperature rise and improved juvenile fitness at longer CP illustrate that dormancy of noble crayfish eggs represents a form of true diapause that is regulated solely by temperature and can be accelerated beyond a threshold only at the expense of higher mortality and reduced juvenile fitness.

Introduction

There are several biological constraints to productivity in cold-water-adapted astacid crayfish, and overcoming them is critical for economically successful culture. In particular, the long incubation period of astacid eggs is challenging for commercial applications (Huner and Lindqvist 1991). However, as astacid crayfish produce among the largest and longest incubated eggs of all crustaceans (Reynolds et al. 1992), show broad plasticity of egg-incubation time over their natural geographical range as well as species-specific annual variability (Dröscher 1906; Reynolds et al. 1992), a popular approach of astacid culture has been to shorten the period from spawning to hatching by an accelerated incubation of eggs (e.g. Reichenbach 1886; Mason 1977b; Pérez et al. 1998a; Carral et al. 2003) to increase the animals' growth period in the first year (Huner and Lindqvist 1991).

Temperature is the most important abiotic factor for embryonic development and timing of hatching in crayfish (Reynolds et al. 1992; González et al. 2009a). Cukerzis et al. (1978) and later Taugbøl and Skurdal (1995) concluded from the long incubation period that *Astacus astacus* requires a period of cold ambient water temperature to successfully complete embryonic development. Subsequent observational and experimental studies confirmed that a cold period improves survival in signal crayfish *Pacifastacus leniusculus* (Celada et al. 1988; Carral et al. 1992), but information on the optimal temperature regime and cold period for eggs of noble crayfish *A. astacus* is lacking.

Artificial incubation (AI) techniques are considered preferable over maternal incubation for experimental studies (Mason 1977a; 1977b; Carral et al. 2003; González et al. 2009a; Kouba et al. 2013) as they allow decoupling of maternal and embryonic responses to experimental variation. For culture purposes, space and energy requirements are significantly reduced, and survival rates are generally higher in artificial than in maternal incubation (Pérez et al. 1999). Some commercial applications have been presented (e.g. Järvenpää and Ilmarinen 1990; Jones and Valverde 2020), and the development of improved AI techniques and procedures has received considerable attention during the last decade (González et al. 2009a; Jones and Valverde 2020). As most studies focus on signal crayfish *P. leniusculus* (e.g. Celada et al. 1988; González et al. 2009a) and white-clawed crayfish *Austropotamobius pallipes* (e.g. Rhodes 1981; Reynolds et al. 1992; Pérez et al. 1998a, 1998b, 1999) species specific information for *A. astacus* is required.

In addition to incubation efficiency, carry-over effects from embryo to larvae (Ituarte et al. 2019) that may affect the physiological condition of stage II juveniles are important for a viable culture and for successful restocking, but no published information is available about the effects of accelerated incubation under different cold periods during AI on subsequent juvenile crayfish performance for *A. astacus*.

Therefore, we studied the effects of three different extended cold periods on early stripped and artificially incubated eggs of *A. astacus* on embryonic development, hatching patterns and subsequent juvenile crayfish performance.

Material and methods

Origin of crayfish broodstock and eggs

150 female and 50 male noble crayfish from an earthen pond at Oeversee Crayfish Farm (Schleswig-Holstein, Germany) were equally distributed to 10 circular polyethylene tanks (2 m diameter, 0.8 m height) filled with pond water. The density was 10 crayfish per m² and the sex ratio 1 male per 3 female crayfish. Water temperature in the ponds and the experimental tanks was 13.5 °C at the time of stocking (October 8th). The first mating was observed seven days after stocking at a water temperature of 12.5 °C. Mating activities lasted for 35 days until November 16th when the water temperature had dropped to 9 °C. Eggs were removed after the water temperature had dropped below 4 °C for 10 days to 1.5 °C on December 15th following the procedure described by Mason (1977b) using a pair of blunt forceps and sliding them past the tip of the endopodite.

Incubation device and experimental set-up

We used a modified incubation method on a moving tray (Järvenpää and Ilmarinen 1990; Jones and Valverde 2020). The central unit is a moving tray that is driven back and forth by an electric motor at a frequency of 10-12 rounds per minute and an amplitude of 10-15 cm. The tray is fitted with triangular, perforated polyethylene baskets with an effective volume of 60 ml holding up to 200 noble crayfish eggs. Three incubators, each representing one experimental regime, were set up in a temperature controlled room with temperature set at 7.5 °C throughout the experimental period. Thermostat-controlled heating of process water was achieved for each incubator individually by two commercial aquarium heaters situated in tank 3b and thermostats situated in tank 3a (Fig. 1).

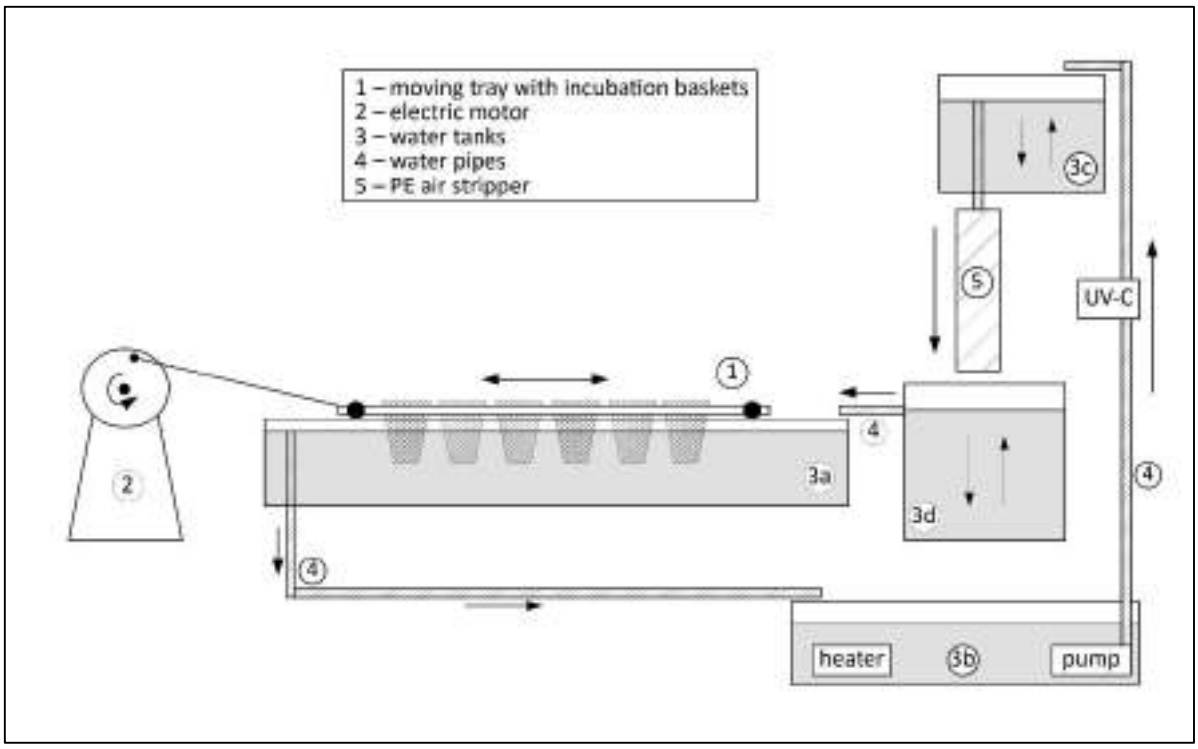

Figure 1: Schematic view of the incubator. Single ended arrows indicate direction of water flow, double ended arrow indicates movement of tray. The wheeled tray (1) is driven back and forth by an electric motor (2) at a frequency of 10-12 rounds per minute and an amplitude of 10-15 cm. The tray is fitted with a user-defined number of triangular, perforated polyethylene baskets. Water level in tank 3a is adjusted to cover the eggs. To prevent oversaturation with volatile compounds and ensure high levels of oxygen saturation, the process water is stripped over a PE structure (5) and well aerated in tanks 3c and 3d. UV-C sterilisation is used to reduce microbial colonisation of process water.

Assessment of incubation efficiency and embryonic development

Eggs were placed into the incubation baskets immediately after stripping and transported to the facilities of Kiel University on wet wood wool within 3 hours. The eggs were kept for 10, 24 and 38 days at 7.5 ± 0.5 °C. After this extended cold period (CP) water temperature was gradually elevated within a week to 17.5 ± 0.4 °C and kept constant until the end of the experiment.

To study embryonic development, 300 eggs from six different females were carefully mixed and equally distributed to six baskets per incubator. The eggs were at the blastosphere stage phase II to III at the time of stripping and had completed approximately 5 % of their development. From each temperature regime, 10 eggs were randomly selected once a week, and their developmental stage was classified according to the relative developmental staging system developed for crayfish by Sandeman R and Sandeman D (1991).

To assess incubation efficiency, a total of 720 eggs were collected from 60 different females. Each incubator was fitted with 10 replicate baskets, and each basket contained 24 eggs

from six different females. Surviving eggs were counted every second day, and dead eggs were removed. Hatching was monitored daily and stage II juveniles were removed daily. Stage II wet weight was determined immediately upon removal or after hardening of the exoskeleton to the nearest 0.1 mg (R160P balance, Sartorius AG, Göttingen, Germany).

Water temperature in each incubator was constantly monitored; oxygen saturation (99.2 ± 2.7 %) and pH (8.58 ± 0.08) were measured every second day using portable devices (Oxi 3310 and pH 3310, WTW, Weilheim, Germany). NO_3-N (< 2 mg/L), NO_2-N (< 0.015 mg/L) and NH_4-N (< 0.03 mg/L) were analysed once a week using LCK tests with a DR5000 UV/VIS Spectrometer (Hach-Lange GmbH, Berlin, Germany).

Assessment of juvenile crayfish performance

We used animals from the ten replicate baskets to assess incubation efficiency. From each temperature regime, juveniles that moulted successfully to stage II were pooled and 80 randomly selected animals assigned to four replicate 112 L aquaria at an initial density of 125 crayfish per m^2. The aquaria were equipped with holed bricks providing structure and shelter, a thin layer of sand, fine gravel and leaf litter (*Acer campestre)*. The experiment took place in a temperature-controlled room at 20 °C and a light:dark regime of 12h:12h. The aquaria were filled with tap water four weeks prior to stocking, and 50 % of the water was replaced once a week. During the experimental period of 100 days, the animals were fed daily with an equal mixture of two formulated diets (Kråkebolle and Edelkreps, Nofima AS, Tromsø, Norway). Once a week, frozen chironomid larvae were also provided.

Water temperature (20.1 ± 0.3 °C), oxygen saturation (93.1 ± 3.2 %) and pH (8.23 ± 0.21) were measured twice a week using portable devices (Oxi 3310 and pH 3310, WTW, Weilheim, Germany). NO_3-N (6.2 ± 2.3 mg/L), NO_2-N (< 0.015 mg/L) and NH_4-N (< 0.03 mg/L) concentrations were analysed once a week using LCK tests with a DR5000 UV/VIS Spectrometer (Hach-Lange GmbH, Berlin, Germany).

Wet weight and total length were determined prior to stocking to assess initial individual weight and length. After 100 days, all surviving animals were counted and weighed to the nearest 0.1 mg on a Sartorius R160P. To determine total length, crayfish were placed on a grid, photographed and total length measured using graphic software (gimp 2.6.11). Specific growth rate (SGR) is expressed as proposed by Evans and Jussila (1997):

$$SGR = \ln (W_f) – \ln (W_i) / t * 100$$

where W_i is the initial individual weight, W_f is the final individual weight and t is the time period of the study.

Data analysis

Differences in incubation efficiency, stage II wet weight and juvenile crayfish survival were analysed using one-way Analysis of Variances (ANOVA) with *p* values based on Tukey-HSD or, in case of unequal sample sizes, on Tukey-Kramer post-hoc test. Differences were considered significant at $p < 0.05$. Survival data was arcsine transformed prior to running the statistical test. All statistical analyses were performed using SPSS 27. Graphs were created using SigmaPlot™ 12.3.

Results

Embryonic development

Temperature rise was initiated at day 10, 24 and 38 of artificial incubation (AI), and nominal temperature was reached six days later at day 16, 30 and 44. Accelerated embryonic development did follow temperature rise with a delay of a few days (10-day CP) or immediately (24-day CP), but was observed prior to temperature rise for eggs exposed to 38-day CP (Fig. 2).

The difference in hatching term was similar to the differences in CP for 24- and 38-day CP, but was four days shorter than expected for animals in 10-day CP. Similar observations were made for moulting to stage II. The total number of degree days required for completion of embryonic development increased with increasing CP from 1114-1255 degree days in 10-day CP to 1226-1327 degree days in 38-day CP. Synchronization of hatching was improved by 38-day CP with hatching and moulting equally limited to 7 days and no overlap between both. In contrast, hatching took place over 11 days (10- and 24-day CP), and the hatching period did overlap with the moulting period.

We observed the formation of five conjoined twins in early developmental stages in 10- and 24-day CP. Two conjoined twins developed and hatched successfully (Fig. 3), but died during moulting.

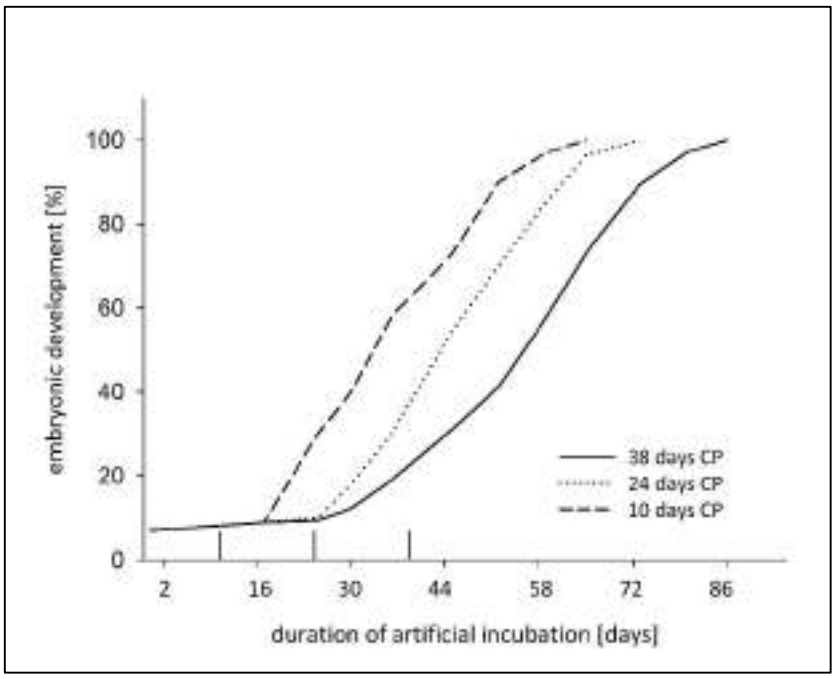

Figure 2: Embryonic development until appearance of hatchlings in response to three different cold periods (CP) during artificial incubation. Vertical bars on the x-axis indicate time points of temperature rise.

Figure 3: Conjoined twins of the 'duplicitas completa' (left) and 'duplicas anterior' (right) type (Scholtz 2020) that successfully developed and hatched in 10-days cold period but died during moulting to stage II.

Incubation efficiency

Length of CP affected hatching success, stage II survival rates (ANOVA both $F_{2,27} > 9.110$, both $p < 0.001$) and stage II wet weight (ANOVA $F_{2,26} = 5.120$, $p = 0.013$). A CP of 24- and 38-days had a positive effect on hatching success, stage II survival and stage II wet weight (all $p < 0.05$) but no parameter did differ between 24- and 38-day CP (all $p > 0.165$) (Fig. 4).

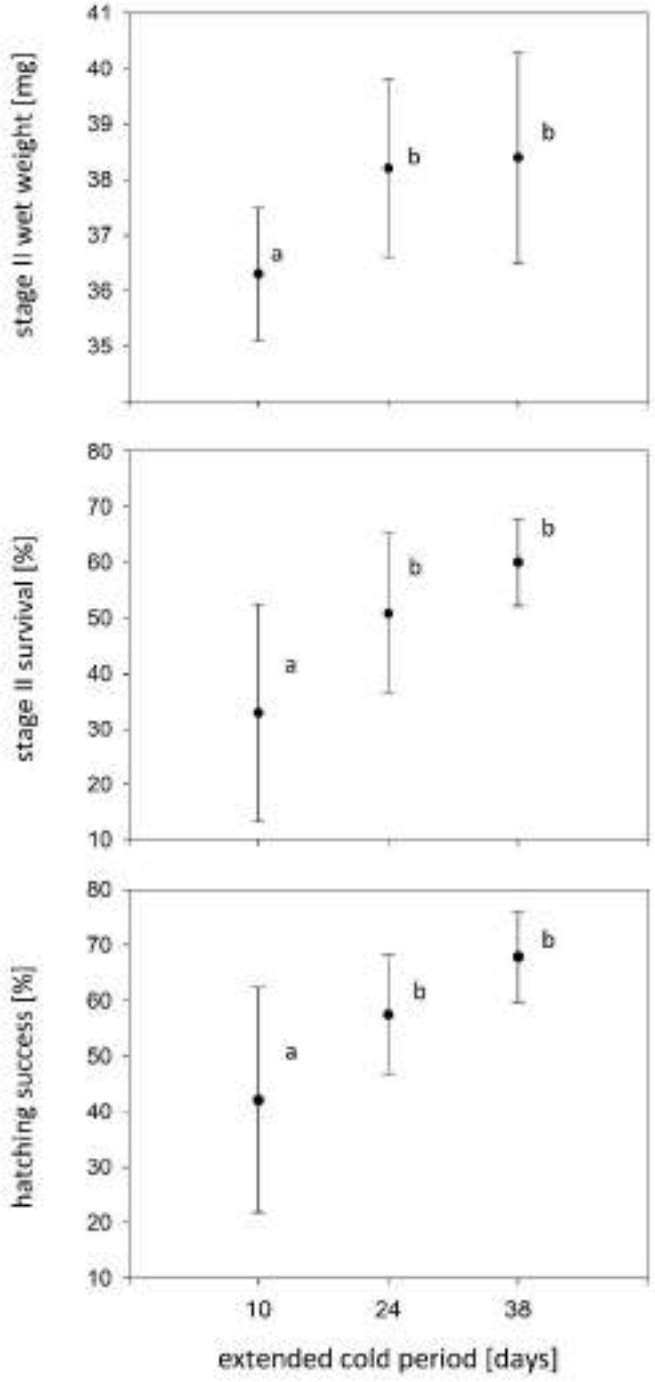

Figure 4: Parameters of incubation efficiency in response to three different cold periods during artificial incubation. Dots display means of 10 replicate incubation baskets and error bars display standard deviation. Different indexed letters indicate significant differences (ANOVA, Tukey-HSD $p < 0.05$).

Mortality due to moulting was 25.8 ± 16.9 % (10-day CP), 13.2 ± 11.7 % (24-day CP) and 11.5 ± 6.8 % (38-day CP) relative to successful hatchlings and 9.2 ± 5.5 % (10-day CP), 6.7 ± 4.5 % (24-day CP) and 7.9 ± 5.4 % (38-day CP) relative to the initial number of eggs. CP affected mortality due to moulting relative to successful hatchlings (ANOVA $F_{2,26}$ = 3.732, p = 0.038) but moulting mortality relative to initial number of eggs was not significant (ANOVA $F_{2,27}$ = 0.746, p = 0.484).

Eggs in all temperature regimes experienced three periods of higher mortality (Fig. 5). Approximately 10 % mortality occurred during the first 10 days of AI after stripping. Another period of increased mortality followed the beginning of accelerated embryonic development. The last period of increased mortality was observed prior to and at hatching.

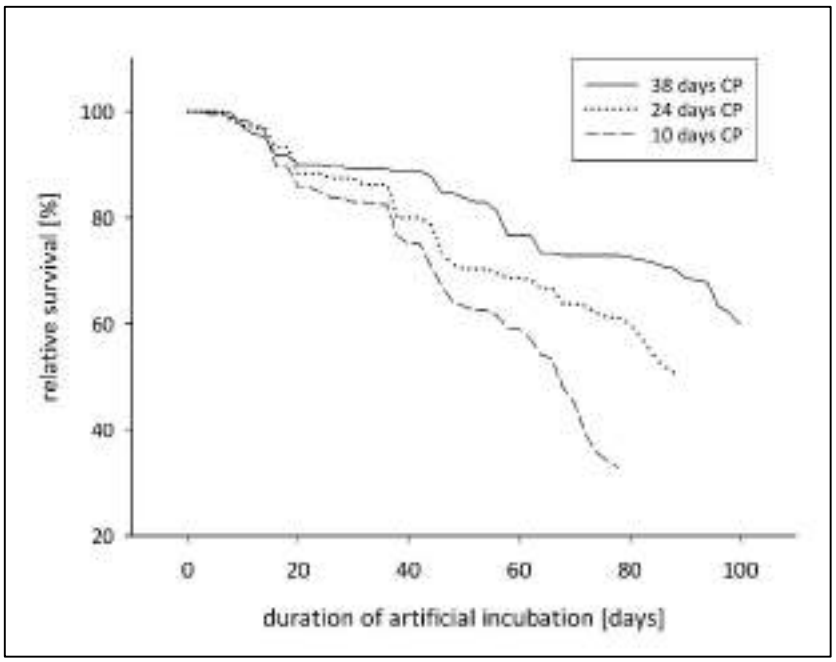

Figure 5: Mean relative embryonic survival (n=10) over the period of artificial incubation. Eggs in all temperature regimes experience three periods of higher mortality that are followed by periods of lower mortality. Higher mortality occurs in response to stocking, to a rise in temperature (day 10, 24, 38) and prior to and at hatching.

Juvenile crayfish performance

Survival was high for crayfish from all temperature regimes and did not differ significantly (ANOVA $F_{2,9} = 1.381$ $p = 0.300$). Final wet weight and SGR were highest for animals that developed under 38-day CP, but differences were not significant (ANOVA both $F_{2,9} < 2.110$, both $p > 0.177$, Tab. 1).

Table 1: Performance of groups of 20 juvenile crayfish that hatched after artificial incubation under different cold periods (CP) and were subsequently reared under identical conditions for 100 days; mean ± SD (n = 4), SGR = specific growth rate.

	10 days CP	24 days CP	38 days CP
Survival [%]	85.0 ± 9.2	90.0 ± 4.1	92.5 ± 2.9
Mean wet weight [mg]	281.1 ± 30.2	293.8 ± 31.8	331.7 ± 40.9
Minimum wet weight [mg]	79.7	119.0	98.9
Maximum wet weight [mg]	794.4	812.3	1047.1
SGR [% / day]	1.88 ± 0.11	1.91 ± 0.11	2.03 ± 0.12

Discussion

Embryonic development and hatching synchrony

The majority of embryos developed normally in all temperature regimes. Notably, we observed the formation of five conjoined twins from early embryonic development. Within the Crustacea, conjoined twins mostly occur in decapods (Scholtz 2020) and have also been reported in crayfish (e.g. Harlioğlu 1996; Alwes & Scholtz 2006). Double anterior structures as observed once in our study have yet only been reported by Rudolph and Martínez (2008) and the causes and mechanisms for the formation of conjoined twins are not fully understood (Scholtz 2020). Even though all conjoined twins were observed after temperature rise and in 10- and 24-day cold period only, our study design does not allow to strictly assign their development to the experimental conditions. One can speculate, however, that the temperature difference between the broodstock holding tanks (1.5 °C) and the incubation devices (7.5 °C) at the time of or shortly before gastrulation may have favoured the formation of the relatively large number of conjoined twins in our study.

Diapause is generally scarce in the Malacostraca (Hairston and Cáceres 1996) and hardly any information is available on its effects on hatching synchrony in decapods. However, diapause is known to synchronise hatching in Branchiopoda (De Stasio 2004; Baumgartner and Tarrant 2017) and Maxillopoda (Stross 1966; Alekseev 2007). As astacid crayfish hatch at the decapodid stage without a pelagic larval phase (Anger 2001) and juveniles start feeding after the first moult (Reynolds 2002), asynchronous hatching may result in significant mortality due to predation of early hatched stage II juveniles on their late hatched conspecifics as observed by Mason (1977a) and Melendre et al. (2007) for *Pacifastacus leniusculus* and recently reported by Shun et al. (2020) for *Cherax quadricarinatus*. Therefore, a highly synchronous hatching is desirable to facilitate large-scale application of artificial incubation (Jones and Valverde 2020), but current knowledge on abiotic effects on hatching synchrony is largely based on unspecific field and laboratory observations (Rhodes 1981; Taugbøl and Skurdal 1990). In our study, all eggs were obtained from a single population and were maintained under identical environmental conditions prior to the experiment. Even though we did not monitor spawning, mating was observed over a period of 35 days. The spread of hatching was limited to 11 days in medium and short CP and was further shortened to 7 days in 38-day CP, which is consistent with the observations on maternal incubation by Taugbøl and Skurdal (1990) for *Astacus astacus* from Norwegian lakes. Improved synchrony at 38-day CP resulted in the absence of an overlap of hatching and moulting period. It

is apparently independent of maternal effects as well as independent of chemical or behavioural interactions between the late embryo and the female and depends solely on temperature regime. This illustrates the potential of our temperature regime for large scale applications.

Incubation efficiency and juvenile crayfish performance

Our results demonstrate clear effects of temperature regime on incubation efficiency and stage II wet weight with 38-day CP generating the best results for all assessed parameters (Fig. 4). Higher mortality and lower stage II juvenile wet weight in shorter CP can be related to the exhaustion of metabolic reserves during diapause and temperature induced accelerated embryonic development (Reynolds 2002; García-Guerrero et al. 2003). Cannibalism likely was of minor importance for mortality as we removed newly moulted stage II juveniles daily (Melendre et al. 2007).

The significance of a cold period for successful embryonic development has been demonstrated for *P. leniusculus* (Celada et al. 1988; Carral et al. 1992) and *Austropotamobius pallipes* (Rhodes 1981; Policar et al. 2009). Given suitable incubation conditions, the time of egg stripping is of minor importance (Pérez et al 1998b; Carral et al. 1992) and optimal duration of diapause is similar in astacid crayfish. More than 45 days CP did not improve results in *A. pallipes* (Policar et al. 2009) and in *P. leniusculus* high survival was obtained by a three to four weeks CP in maternal (Celada et al. 1988) and artificial incubation (Carral et al. 1992). In agreement with these observations, our results show that an extended 28-days CP (i.e. 38-days total CP) is sufficient to achieve high incubation efficiency in *A. astacus*. The optimal time point for entering diapause, however, seems to differ between *P. leniusculus*, *A. pallipes* and *A. astacus*. For *P. leniusculus*, the optimal time point for entering diapause was stage IX when the embryo had developed naupliar appendages (Celada et al. 1988), whereas entering diapause at this point of embryonic development did not have positive effects on embryos of *A. pallipes* (Celada et al. 2001). For *A. astacus* in our study, prolonged diapause at the blastosphere stage II-III positively affected incubation efficiency similar to the observations of Rhodes (1981) for *A. pallipes*. This difference between species may be explained by their life-cycle under natural conditions. *A. astacus* and *A. pallipes* reproduce at temperature between 9-12 °C while mating and spawning in *P. leniusculus* takes place several weeks earlier at temperatures around 15 °C (Reynolds et al. 1992). Therefore, it is likely that eggs of *A. astacus* and *A. pallipes* are adapted to enter diapause at earlier developmental stages than those of *P. leniusculus*.

If accelerated AI is applied in culture, physiological condition of stage II juveniles and subsequent growth may be of even greater importance than incubation efficiency. Only if early hatched animals perform similar to (Sáez-Royuela et al. 1995) or even better than their late hatched conspecifics (González et al. 2009a) the advantage of a longer growth period can be converted into higher biomass at the end of the growing season. Policar et al. (2004) report slower growth rates for maternally incubated noble crayfish juveniles that hatched at elevated temperature. Overall, growth rates at the end of our 100-day rearing period were similar to those achieved by Policar et al. (2004) and animals from the longest CP of 38 days yielded largest individual but effects on survival and growth were not significant. It should, however, be considered that temperature-induced accelerated incubation does not only affect incubation efficiency, but may as well influence juvenile performance highlighting the importance of a species-specific incubation regime for successful culture.

In conclusion, our data support the hypothesis that egg dormancy of *A. astacus* represents a form of true diapause that can be accelerated beyond a threshold only at the expense of higher mortality and reduced juvenile fitness (Alekseev and Starobogatov 1996). Higher synchronization of hatching as well as no overlap between hatching and moulting period at 38-day CP imply benefits for commercial application of accelerated AI such as exact planning of stocking and reducing the need for daily stage II juvenile removal.

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CHAPTER II

RNA/DNA RATIO IS AN EARLY RESPONDING, ACCURATE PERFORMANCE PARAMETER IN GROWTH EXPERIMENTS OF NOBLE CRAYFISH *ASTACUS ASTACUS* (L.)

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aquaculture, physiological indicator, intermittent feeding, feeding experiment, zooplankton

Abstract

Recent intensification efforts of astacid culture considerably depend on the appropriate assessment of the animal's physiological condition both for research and application. We conducted a four weeks feeding experiment to assess temporal resolution and accuracy of different response parameters (RNA/DNA ratio, RNA per wet weight, carapace length, wet weight, specific growth rate). Juvenile noble crayfish were exposed to five feeding regimes that differed in feeding frequency and food availability. Continuous growth was detected in all feeding regimes with individual increase up to 90 % (wet weight) and 17 % (carapace length), respectively. Morphometric parameters allowed separation of three weight-groups or two length-groups. During the experimental period RNA/DNA ratios showed both decrease (-17 %) and increase (+35 %), with superior accuracy than morphometric parameters, separating four groups. Based on RNA/DNA ratios different feeding regimes were detected earlier, with two groups separated already after 3 weeks. RNA/DNA ratio was clearly superior to RNA per unit wet weight, as the latter failed to detect any differences between groups. In conclusion, RNA/DNA ratio is a valuable tool in nutritional studies with freshwater crayfish if overall growth is the key variable of interest.

Introduction

Exploitation of freshwater crayfish stocks has a long tradition in Europe (Ackefors and Lindqvist 1994; Swahn 2004) and currently, table size crayfish represent a luxury food item of high commercial value. Often, market demands for noble crayfish *Astacus astacus* (Linnaeus) cannot be satisfied because natural populations are declining due to the dispersion of the crayfish plague *Aphanomyces astaci*, habitat loss and competition with non-indigenous species (Westman and Savolainen 2001; Jussila and Mannonen 2004). In the last decades, production of noble crayfish has been practised in semi-intensive pond systems. Recently, rising demand for both (re-)stocking material and especially table size crayfish has led to novel attempts to intensify culture in Europe. Those primarily focus on introduced signal crayfish *Pacifastacus leniusculus* (Dana) and native noble crayfish *A. astacus* (e.g. Kouba et al. 2010, González et al. 2010). The latter species is more economically auspicious, as animals can be brought to market for both stocking and consumption purposes. Production of table size crayfish requires intensification of culture, which depends considerably on the appropriate assessment of the physiological condition of an individual (e.g. well-fed or starving).

Researches promoting culture intensification in crayfish apply somatic growth rate described as an increase in wet weight or body length. However, high individual variation within an age group along with intermittent growth (related to the moulting cycle) result in a restriction of temporal resolution and loss of accuracy.

As alternative approach, various biochemical indicators to assess physiological condition have been applied. Weber et al. (2003) suggest, that a strict combination of various biochemical indices representing a certain aspect of the animals' physiological condition allows accurate assessment of condition and growth potential of wild fish. These indices include whole body total lipids and triglycerides, muscle RNA/DNA ratio and muscle protein. Similar conclusions are drawn by Gilliers et al. (2006) for assessing habitat quality of nursery grounds for juvenile fish based on a combination of different bio-indicators that were measured on individual fish. However, when looking at overall growth capability, quantification of nucleic acids has great potential, as those have a major function in growth and developmental processes. Protein biosynthesis, which may lead to changes in somatic growth, is regulated over two pathways: either increase in the amount of RNA or increased ribosomal activity (Clemmesen 1994b; Malzahn et al. 2003). Even though increased ribosomal activity may uncouple the positive relationship between RNA concentration and protein biosynthesis, measurements of RNA or DNA per unit wet weight or per individual

(Wagner et al. 2001; Desai and Anil 2002) or RNA/Protein ratio (Moss 1994a/b; Parslow-Williams et al. 2001) are successfully used. For fish larvae (Clemmesen 1993; 1994a) and crustaceans (Moss 1994a/b; Wagner et al. 2001; Parslow-Williams et al. 2001; Desai and Anil 2002), RNA/DNA ratio has been shown to be a good indicator of their nutritional status. Also Wolf (2004) and Stumpf et al. (2011) used RNA/DNA ratio to assess physiological condition of juvenile crayfish. In adult noble crayfish this parameter has been applied in a field survey to estimate individual growth rates (Olsson et al. 2008). Though RNA/DNA ratio reflects capacity of protein synthesis normalised to DNA content, to some extent it still is weight and stage dependent, due to metabolic (protein production rate and amount of active ribosomes) and physiological changes (e.g. white muscle protein to body mass ratio) (Ciotti et al. 2010). Nevertheless, within a developmental stage RNA/DNA ratio is known to reduce noise caused by individual variation in body size and it has been shown to be a reliable parameter (Moss 1994a/b; Wagner et al. 2001; Desai and Anil 2002; Vrede et al. 2002). In a recent review, Koop et al. (2011) suggest RNA/DNA ratio as an instantaneous and less time consuming method for measurements of invertebrates' physiological condition in the field.

We investigated the feasibility of RNA/DNA ratio as an alternative performance parameter in juvenile *A. astacus*. Since food supply is an effective factor for controlling somatic growth and has direct effect on the organism's physiological condition, we designed an experiment with different feeding regimes. Using this setup, we compared the relationship between somatic growth (carapace length, wet weight, specific growth rate) and nucleic acid based indicators (RNA/DNA ratio, RNA per unit wet weight). We expected RNA/DNA ratio to reflect chosen feeding regimes more accurately and with higher temporal resolution than the standard morphometric parameters carapace length and wet weight. Further, using juveniles with a high moulting frequency and higher growth rates (e.g. Kouba et al. 2010) allows shorter experimental periods. The fluorimetric measurement of RNA content and RNA/DNA ratio established by Clemmesen (1993) and modified by Malzahn et al. (2003) has frequently been applied and therefore was our method of choice.

Materials and methods

Experimental set-up and study animals

We used four weeks old juvenile crayfish obtained from crayfish eggs incubated under controlled conditions (for details see Järvenpää 1990; Jeske 2007). Until starting the experiment, the animals were kept in communal tanks under experimental conditions in a temperature controlled room at 12h:12h light dark cycle at Kiel University, Department of Limnology.

The experimental system consisted of 10 recirculating 112 L tanks filled with conditioned tab water (19.0 ± 1.1 °C, pH 8.4 ± 0.1, 9.2 ± 0.1 mg/L O_2, 6.0 ± 0.2 mmol/L alkalinity). Each tank was equipped with five 2.5 L net-cages (mesh size 0.6 mm) housing the animals. Structure and shelter were provided by woody brown coal (Xylit, Vattenfall Europe Mining AG) added to the net-cages. Five feeding regimes with 10 replicates were set up in randomised block design with each 112 L tank representing one block.

Prior to stocking, wet weight (WW) of all individuals was determined (Sartorius balance R160P) and carapace length (CL) measured using an eyepiece micrometer with a Zeiss stereo microscope (Stemi SV11). 400 juvenile *Astacus astacus* (WW 45.65 ± 2.56 mg, CL 6.95 ± 0.15 mm) were stocked in groups of eight randomly selected individuals into the net-cages at an initial density of 400 Ind./m².

The animals were fed frozen *Daphnia* sp. bred at the outdoor facilities of the Department of Limnology. *Daphnia* sp. were caught every third day and feeding portions than were kept frozen in -20 °C. The five feeding regimes differed in feeding frequency (feeding 1 fed once a week, feeding 2 fed twice a week up to feeding 5 which was fed five times a week) and therefore in the amount of food available. One feed unit was equal to 4 % of the wet weight of stocked crayfish (Chybowski 2007). Once a week, the amount of food was adjusted for growth, dead and removed individuals. After four weeks the experiment was terminated.

Assessment of performance parameters

We used five different parameters to assess physiological condition of crayfish in response to feeding regime. One individual from each of the 10 replicate net cages was removed weekly, thereby equally reducing density in all net-cages to 200 Ind/m² at the end of the experiment. WW and CL were determined immediately and the animal frozen (-80°C) for further analysis. Analysis

of RNA and DNA contents were conducted at the facilities of GEOMAR-Helmholtz-Zentrum für Ozeanforschung in Kiel, applying a modified fluorescence technique (Clemmesen 1993; Malzahn *et al.* 2003).

From the frozen or defrosted individual a small amount of tissue (approx. 1 mg) was removed from the ventral side of the abdominal muscle, weighed and returned to -80°C until further use. For extraction of nucleic acids, 400 µL Tris EDTA-SDS 0.01 % buffer and some glass-beads were added to the tissue-sample and homogenised in a shaking-mill (type: MM2, Retsch GmbH & Co.KG) for 20 minutes at room temperature. Samples were centrifuged at 3830 g and 0°C for 8 minutes (type: 3-18K, Sigma). 300 µL of the supernatant (homogenate containing nucleic acids) were transferred to a new tube and the precipitate was discarded. All fluorimetric measurements were made using a fluorimeter (Fluoroskan Ascent, type 374, Labsystems) and a 96-well micro titre plate with ethidium bromide as specific nucleic acid fluorescent dye (Clemmesen 1993). We used 16S and 23S ribosomal RNA from *E. coli* as RNA standard (average slope ratio: 30.92 ± 1.36 (n=10), 0.25 mg/mL, Boehringer, 206938) and λ-phage DNA as DNA standard (average slope ratio: 45.63 ± 1.79 (n=10), 4.0 mg/mL, Boehringer, 745782). Prior to each measurement autofluorescence of the homogenate was determined to correct the DNA and RNA fluorescence. To measure DNA content of homogenates, RNA was broken down using RNase A (from bovine pancreas, Serva). RNA content was calculated as the difference between the corrected fluorescence of the homogenate and corrected DNA-fluorescence (Clemmesen 1993).

As additional WW based parameter, we calculated specific growth rate (SGR) as suggested by Evans and Jussila (1997):

$$SGR = \ln (W_f) – \ln (W_i) / t * 100$$

with W_f = final WW [mg] after four weeks, W_i = initial WW [mg] at time 0 for all feeding regimes, t = time period of experiment [days].

Data analysis

Data was analysed using two-way Analysis of Variances (ANOVA) with feeding regime and week as the two independent variables and WW, CL, RNA/WW or RNA/DNA ratio as the dependent variable, respectively. Normality was analysed using Shapiro-Wilk statistics, Levene-Median Test was used to test for homogeneity of variances and log transformation was applied for WW and RNA/WW to meet the assumptions of the statistical test (i.e. normality, homogeneity of variances). Data for WW violated the assumption of homogeneity of variances despite

transformation. Heteroscedasticity of data is likely to cause type 1 error (i.e. rejecting the null hypothesis even if it's true) but ANOVA is robust to violation of the assumption of homogeneity of variances if sample sizes are equal (Quinn and Keough 2001), which was the case for WW in our study. Therefore, we applied multiple comparisons of two-way ANOVA for log transformed WW data and additionally calculated Kruskal-Wallis one-way ANOVA on Ranks to validate the results. Treatments were compared by Holm-Sidak post-hoc test and differences were considered significant at $p<0.05$. To analyse the cause of the variation found in WW simple linear regressions were used. All analysis was performed using SigmaPlot™ 12.3.

Results

Morphometric growth parameters

During the experimental period a continuous increase in WW and CL was detected. Individuals that were fed more often also had a significantly faster increase for both parameters (Fig. 1). WW increased between 30 % (feeding 1) and 90 % (feeding 5), CL increased between 5 % (feeding 1) and 17 % (feeding 5). Results of the two-way ANOVA indicated a significant main effect of both feeding regime and week on CL and WW (all $p<0.001$), but no interaction between the two factors was detected (all $p>0.423$). However, significant differences between feeding regimes were apparent after four weeks. Based on WW three feeding regimes could be identified (Fig. 1). Feedings 4 and 5 showed a significant increase compared to feedings 1 and 2 ($p<0.05$). Multiple comparisons based on the untransformed, ranked data confirmed these results. When comparing CL (Fig. 1), statistically significant differences between feeding 5 and feedings 1 to 3 ($p<0.05$) were found.

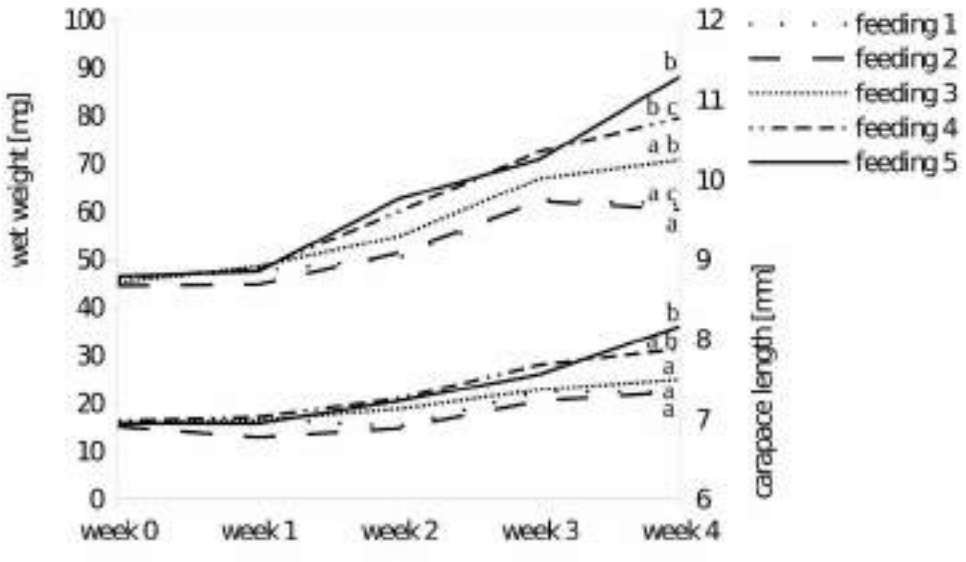

Figure 1: Changes in wet weight (WW) (upper) and carapace length (CL) (lower) in each of the five feeding regimes over duration of 4 weeks. The values are means of 7–10 replicates. Different letters at the end of each line indicate statistically significant differences (two-way ANOVA, $p < 0.05$) at the end of experiment (week 4).

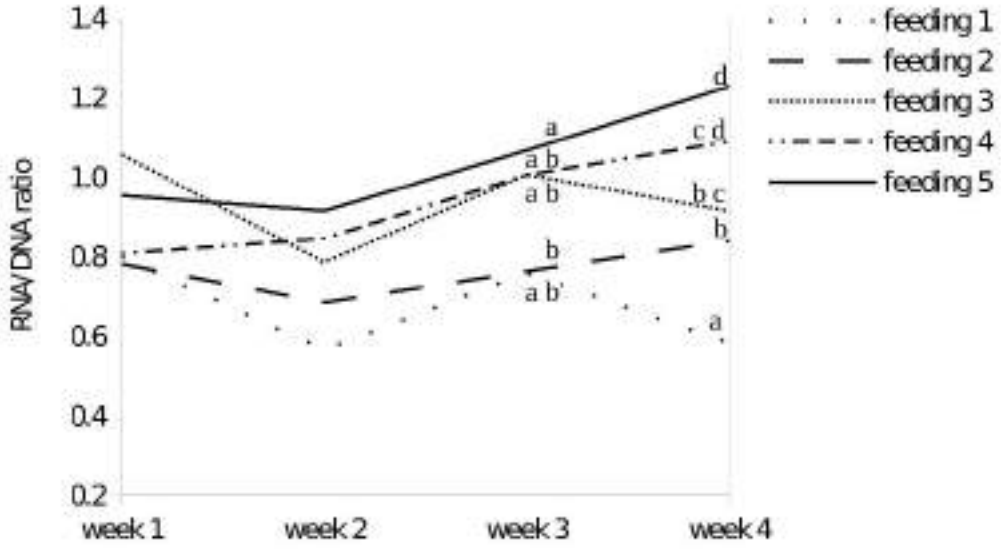

Figure 2: Changes in RNA/DNA ratio in each of the five feeding regimes over duration of 4 weeks. The values are means of 7–10 replicates. Different letters at the end of each line (week 4) or over and under each line (week 3) indicate statistically significant differences (two-way ANOVA, $p < 0.05$).

Nucleic acid based physiological indicators

Individually measured RNA/DNA ratios ranged from 0.33 (feeding 2, week 1) to 1.52 (feeding 5, week 4) with RNA amounts between 0.27 µg/mg WW (feeding 2, week 2) and 1.61 µg/mg WW (feeding 5, week 4) and DNA amounts between 0.52 µg/mg WW (feeding 3, week 3) to 2.11 µg/mg WW (feeding 1, week 4). Over the experimental period changes in RNA/DNA ratio ranged from -17 % to +35 % (Fig. 2) with a decrease in feedings 1 and 3 and an increase in feedings 4 and 5. No differences were observed for feeding 2 between the beginning and end of the experiment (Fig. 2). Results of the two-way ANOVA indicated a significant main effect of feeding regime (p<0.01) and week (p<0.001) on RNA/WW, but no interaction between the two factors was detected (p=0.858). For RNA/DNA ratio, a significant main effect of feeding regime and week (both p<0.001) as well as an interaction between the two factors was found (p<0.05).

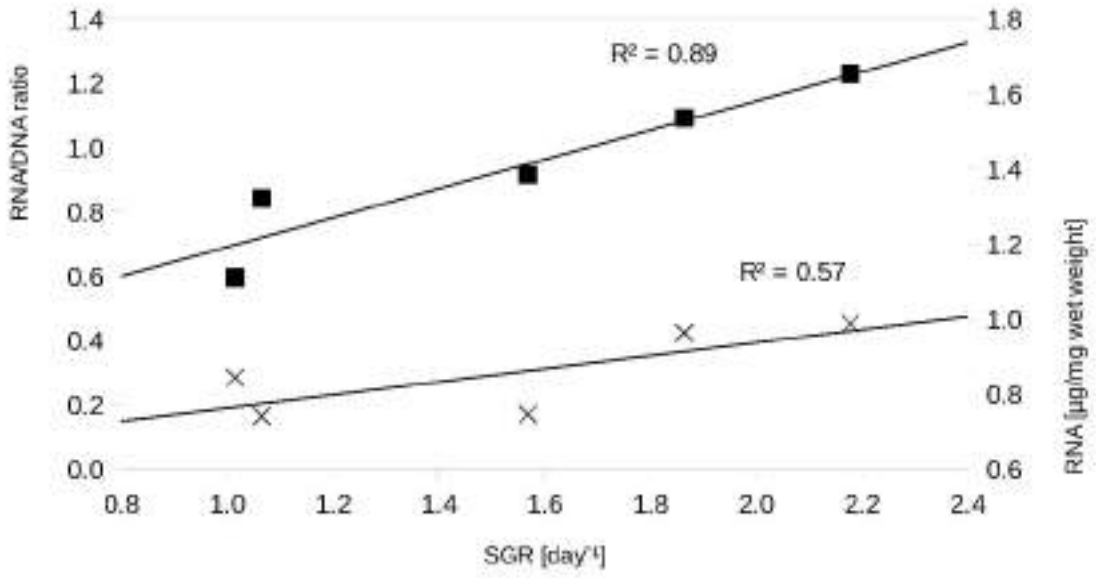

Figure 3: RNA/DNA ratio (squares) and RNA per unit wet weight (crosses) vs. specific growth rate (SGR) for means of each feeding regime at the end of a 4 weeks feeding experiment. The values are means (± SD) of 7–10 replicates. Simple linear regression models: RNA/DNA ratio: y = 0.45x + 0.24, RNA per unit wet weight: y = 0.17x + 0.59.

Accuracy and temporal resolution of response parameters

At the end of the experiment, there were clear differences between the feeding regimes for all response parameters. The means were ascending throughout the feeding regimes with feeding 1 having the lowest and feeding 5 having the highest values (Tab. 1). WW was more accurately reflecting feeding regime than CL, separating three groups, while CL only separated two (Tab. 1).

Calculated SGR values show similar accuracy as CL, detecting only two feeding regimes (Tab. 1). Using RNA/DNA ratio four different feeding regimes could be detected. Comparing the relationship between SGR and RNA/DNA ratio or RNA per unit WW (Fig. 3), simple linear regressions show that RNA per unit WW only explains 57 % whereas RNA/DNA ratio explains 89 % of the variation found in specific growth rate (Fig. 3). Furthermore, RNA per unit WW fails at detecting differences in feeding regime (Tab. 1). RNA/DNA ratio shows differences earlier than the other response parameters (Fig. 1 and 2). WW and CL only indicate differences between treatments in week 4, whereas two feeding groups can be separated based on RNA/DNA ratio already after three weeks.

Table 1: Comparison of different response parameters: carapace length, wet weight, RNA/DNA ratio, specific growth rate (SGR) and RNA per mg wet weight at the end of a 4 weeks feeding experiment with juvenile freshwater crayfish *Astacus astacus*. The values are means (± SD) of 7–10 replicates. The different indexed letters show statistically significant differences (two-way ANOVA, $p < 0.05$).

	Feeding regime				
	1	2	3	4	5
Carapace length [mm]	7.3^{a} (±0.5)	7.3^{a} (±0.3)	7.5^{a} (±0.4)	7.9^{ab} (±0.6)	8.2^{b} (±0.6)
Wet weight [mg]	61.5^{ac} (±9.5)	60.4^{a} (±6.7)	70.7^{ab} (±9.8)	79.5^{bc} (±18.1)	88.1^{b} (±24.3)
SGR [day^{-1}]	1.0^{a} (±0.6)	1.1^{a} (±0.4)	1.6^{ab} (±0.5)	1.9^{ab} (±0.8)	2.2^{b} (±0.9)
RNA/DNA ratio	0.59^{a} (±0.08)	0.84^{ab} (±0.25)	0.91^{bc} (±0.14)	1.09^{cd} (±0.21)	1.23^{d} (±0.08)
RNA [µg mg^{-1} wet weight]	0.84^{a} (±0.33)	0.74^{a} (±0.15)	0.75^{a} (±0.20)	0.96^{a} (±0.26)	0.99^{a} (±0.34)

Discussion

Preference of juvenile crayfish for invertebrate food is well documented (Momot 1995) and several studies have demonstrated enhanced growth rates when the animals were fed a diet including zooplankton (Brown et al. 1992; Verhoef et al. 1998; Parkyn and Collier 2002; Sáez-Royuela et al. 2007; González et al. 2008). Growth rates of juvenile *Astacus astacus* obtained on a diet of *Daphnia* sp. in the presented study are in the range reported by others for juvenile noble crayfish under laboratory conditions (e.g. Kouba et al. 2010), demonstrating the suitability of our experimental design for the animals.

Our results show high accuracy and temporal resolution of RNA/DNA ratio in comparison to carapace length (CL), wet weight (WW) and associated specific growth rate (SGR). Differences found in RNA/DNA ratio between feeding regimes were not temporarily stable but increased with time (Fig. 2) and RNA/DNA ratio was the only parameter that showed an interaction between feeding regime and time of measurement. This indicates the suitability of this method when studying temporal effects of food supply on the growth potential of juvenile noble crayfish, as those changes in RNA/DNA ratio reflect the integrated effects of the last weeks' food supply (Clemmesen 1994b). For CL and WW differences between feeding regimes did also increase with time (Fig. 1) but both response variables did not sufficiently indicate these temporal changes to result in a significant interaction between feeding regime and time of measurement.

RNA/DNA ratio explains 89 % of variation found in specific growth rate (SGR) (Fig. 3). Similar accuracy was found by Moss (1994b) for the white shrimp where RNA/DNA ratio accounted for 80% of the variation in growth rate. A slightly higher correlation value ($R^2 = 0.94$) is reported on RNA/DNA ratio and specific growth rate SGR of *Daphnia* (Vrede et al. 2002) The correlation between SGR and RNA/DNA ratio has been intensively discussed by Ciotti et al. (2010) who emphasise biological (DNA- content of cells) or even methodological (extraction procedure) reasons may lead to the high correlation of SGR and RNA/DNA ratio. Potential bias due to errors associated to laboratory extraction procedure are considered minor due to the fact that RNA and DNA are measured in the same aliquot and are expressed as ratio. The influence of DNA concentration on the correlation seems to be low compared to temperature or developmental stage. Ciotti et al. (2010) conclude that the correlation is a good measure for physiological condition, which could potentially be sensitive to many more factors than can be controlled for. In our experiment the often applied nucleic acid based parameter RNA per wet weight shows no separation of feeding regimes (Tab. 1). However, lack of correlation reported in our study is likely due to the use of wet instead of dry weight in combination with a small tissue sample. In a study by Moss (1994b), RNA per wet weight was well correlated to growth rate and accounted for 76 % of variation found in growth rate. Nevertheless, as indicated above, RNA/DNA ratio performed even better. Interestingly, Parslow-Williams et al. (2001) found the highest correlation to growth rate of lobsters in RNA per dry weight. This parameter performed better then RNA/Protein and RNA/DNA ratio. One could assume that for small tissue samples the use of RNA per dry weight is superior to RNA per wet weight since the adhering water might be a source of error in the latter.

The separation of four feeding groups after four weeks (compared to three or two groups separated by WW and CL, respectively) indicates that RNA/DNA ratio reflects the feeding

regime more accurately than WW and CL. It also shows that the variation found in WW and CL does not affect the measurement of RNA/DNA ratio to a greater extend then the actual feeding regime does. Nevertheless, this parameter probably does not completely eliminate the effect of body size or developmental stage (Clemmesen 1994a; Malzahn et al. 2003). Clemmesen (1994a) showed that herring larvae needed to reach a size of about 30 mm to show no length dependency in their RNA/DNA ratios. Due to the fact that DNA concentration is inversely related to cell size, metabolic or physiological developments may affect RNA/DNA ratios. According to Wolf (2004), the measurement of RNA/DNA ratios is a reliable method for measuring the physiological response of signal crayfish to changes in their metabolism. However, he also suggests that the best time for the measurement would be the most stable intermoult period, since during moulting water absorption into the cells might change the ratio between DNA content and cell size. Wagner et al. (2001) were able to show a stage dependency of the RNA/DNA ratio in *Calanus finmarchicus* (Gunnerus, 1770) and suggested a calibration for each stage. We measured individuals at different time points rather than comparing different developmental stages and did not undertake calibrations concerning developmental stage or moulting period. Faster increases in WW and CL at week 2 and week 3 (Fig. 1) indicate that moulting has most likely taken place during the experimental period. Furthermore, parts of the exoskeleton were found more frequently in the net cages during these two weeks. Whether changes in RNA/DNA ratios (Fig. 2) are also due to the moulting events could not be determined, as moulting was not measured in our study. Even without taking stage dependent adjustments into account, we were able to reach high accuracy and temporal resolution with this method after a short experimental period (4 weeks). Consequently, we conclude that comparison of individuals of similar developmental stage based on relative changes in RNA/DNA ratio between treated or observed groups is feasible.

Temperature is another important and widely discussed variable (Buckley et al. 2008; Ciotti et al. 2010) that influences metabolic activity by principally affecting RNA concentrations. Therefore, RNA/DNA ratios as well as the relationship between RNA/DNA ratio and SGR are modified by temperature. Growth models, which include temperature and interaction terms (temperature with RNA/DNA ratio) have been suggested for different species (Wagner et al. 2001; Buckley et al. 2008; Ciotti et al. 2010). We did not consider this temperature dependency of SGR-RNA/DNA relationship, since individuals reared at suitable temperatures (Kouba et al. 2010) are comparable among each other (Buckley et al. 2008). Therefore, we suggest the effects of temperature on our results can be neglected and food supply had the main influence on the assessed performance parameters.

Furthermore, for stock assessment and field monitoring this method has great potential (reviewed in Koop et al. 2011), even though calibration to developmental stage may be necessary. Many authors have reported age dependent within species variation (Wagner et al. 2001; Lemos et al. 2002; Desai and Anil 2002), but as shown, within an age group RNA/DNA ratio correlates very well with food supply and therefore may be a valuable tool to assess physiological conditions related to food availability of either wild or farmed stocks of freshwater crayfish. For this purpose, an exact and minimal invasive measurement of the animal's physiological condition is desirable. Clear advantages of using RNA/DNA ratio are its accuracy and sensitivity allowing to process tissue samples down to 10 µg dry weight (Clemmesen 1994b). Further, changes in the amount of RNA can be measured rather quickly within 24 hours for some crustacean species (Moss 1994b). According to Clemmesen (1994b) limits for significantly distinguishable changes within the RNA/DNA ratio are, depending on species, 4 to 9 days. Due to this time lag, measurements do not show the effects of the last diet provision, but rather the integrated effects of the last weeks' food supply. This time period is shorter than one moulting cycle in juvenile noble crayfish, thus showing physiological changes earlier than measurements of WW and CL would.

Another important benefit of applying RNA/DNA ratio to assess physiological condition arises for research related to crustacean culture. A major objective for intensive culture lies in the development of adequate feeds, highlighted by numerous studies on this topic (e.g. Ackefors et al. 1992; D'Agaro 2004; González et al. 2008; Garza de Yta et al. 2012; Saoud et al. 2012). But with few exceptions (e.g. Wolf 2004) those are relying on weight increase as a measure of somatic growth rate, thereby necessitating long (several weeks to months) experimental duration. Likely therefore, the vast majority of studies is dealing with juvenile crayfish, as adults will moult less often, requiring even longer experiments. We can show that RNA/DNA ratio correlates very well with food supply for crayfish. Since the method is useful to show ongoing anabolism without needing a moulting event, its application will facilitate shorter experiments and consequently more thorough testing of feeds which is especially important when aiming at producing table size crayfish.

Several controversial findings to whether RNA per weight, RNA/Protein or RNA/DNA ratio is the most accurate performance parameter have been published (Clemmesen 1993; Moss 1994b; Parslow-Williams et al. 2001; Wagner et al. 2001; Desai and Anil 2002). Based on our experimental study we suggest that for noble crayfish, RNA/DNA ratio is an accurate performance parameter that responds rapidly to differences in food supply.

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CHAPTER III

NURSERY OF NOBLE CRAYFISH *ASTACUS ASTACUS* (L. 1758): EFFECTS OF STOCKING TIME AND LIGHTING ON PERFORMANCE OF EARLY-HATCHED JUVENILES

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Abstract

Nursery of noble crayfish can greatly enhance growth period of noble crayfish in the first growing season and suitable lighting conditions are crucial for its successful implementation. Lighting may strongly affect growth of periphyton which is important in the diet of omnivorous crayfish. We studied the effect of different lighting methods (natural sunlight, fluorescent light tubes, light emitting diodes) on performance of juvenile noble crayfish in two consecutive experiments (early spring 28th of March until 20th of May, late spring 21st of May until 18th of July) with temperature of process water dependent on ambient conditions and elevated by a greenhouse. Temperature increased from 12.7 to 17.8°C in early spring and ranged from 18.2 to 23.7 °C in late spring. Survival was higher in late (80.8 ± 3.7 % – 90.4 ± 2.8 %) than in early spring (77.4 ± 7.7 % – 78.7 ± 5.1 %) and not equally affected by lighting. Natural sunlight yielded highest specific growth rates (early: 2.39 ± 0.08 %*day^{-1}, late: 4.53 ± 0.13 %*day^{-1}) and final weights (early: 121.9 ± 4.9 mg, late: 553.6 ± 42.7 mg) of crayfish in both trials. The importance of naturally occurring food items differed between natural sunlight and the other treatments, periphyton characteristics can partly explain differences in crayfish performance. Our results illustrate the potential of periphyton as a complementary food source in nursery of noble crayfish and show that its growth can be promoted by novel lighting techniques.

Introduction

The noble crayfish *Astacus astacus* (L.) is a highly appreciated aquaculture product that is considered superior to other crayfish species and therefore constantly commands very high prices at the market (Holdich 1993; Jussila and Mannonen 2004; Füreder 2016). Harvest from natural stocks cannot support market demands. Consequently, its culture is of growing interest for both restocking and human consumption (Policar and Kozàk 2015). Intensive year-round culture, even though biologically feasible, has frequently proven unprofitable (Ackefors et al. 1992; Jussila 1997; Skurdal and Taugbøl 2002) but semi-intensive pond culture is established in many European countries (Policar and Kozák 2015). There are, however, several biological constraints to an intensification of semi-intensive astacid culture. In particular, high juvenile mortality (Pursiainen et al. 1983), slow growth rates (Kouba et al. 2010) and the seasonal growth pattern of astacid crayfish (Reynolds 2002) make commercial production of noble crayfish challenging.

The implementation of a nursery phase has received considerable attention in culture of warm-water crayfish (Geddes et al. 1995; Parnes and Sagi 2002; Garca de Yta 2009; Ghanawi and Saoud 2012; Jones and Valverde 2020) as juveniles require less space and show higher growth rates compared to adults. Such a nursery has great potential in aquaculture of cold-water astacids, if it extends the growth period of the animals in the first year (Huner and Lindqvist 1991; Ackefors and Lindqvist 1994). This can be achieved by an accelerated incubation of crayfish eggs (Reynolds et al. 1992; Skurdal and Taugbøl 2002) thereby producing early-hatched juveniles several months before hatching would occur under natural conditions (e.g. Hessen et al. 1987).

Optimum water temperature for rearing of noble crayfish juveniles is between 16 and 24 °C (Kouba et al. 2010; Füreder 2016). Consequently, if early-hatched juveniles are produced for nursery in early spring, their subsequent rearing requires heating of process water until ambient outdoor conditions are suitable for pond stocking. As heating of process water is costly, production-scale nursery of noble crayfish usually takes place under ambient outdoor conditions during summer months (Keller 1988; Policar and Kozák 2015), thereby losing its potential to double the animals' growth period in the first year. To overcome unfavourable ambient temperatures, cost-effective and heating of process water by integrating a greenhouse is frequently applied in aquaponics and aquaculture of fish (Zhu et al. 1998; Jana et al. 2019; Akidiva et al. 2020; Alkhalidi et al. 2020), but despite its potential has not received much attention in rearing of cold-water astacid crayfish.

In intensive nursery systems, mortality and reduced growth rates caused by cannibalism and agnostic interactions pose a great challenge to freshwater crayfish production (Taugbøl and Skurdal 1992; González et al. 2011; Kouba 2011; Romano and Zeng 2017). To solve these problems, suitable lighting conditions are crucial (González et al. 2011; Franke et al. 2013) as lighting influences behavioural patterns of crayfish and change the quality and quantity of agnostic interactions (Farca-Luna et al. 2009). For noble crayfish, a clear nocturnal activity pattern can be induced by artificial lighting thereby enhancing survival and growth under culture conditions (Franke et al. 2013; 2015). Different white light spectra do not affect performance of noble crayfish summerlings (Abeel et al. 2016). However, photoreceptors of juvenile crayfish experience an ontogenetic shift in spectral sensitivity during their first weeks in life (Fanjul-Moles and Fuentes-Pardo 1988) thus results obtained for older animals may not be applicable for early juveniles.

Naturally occurring food items enhance growth and survival in cultured warm-water crayfish (Viau et al. 2012; Jin et al. 2019a). In addition to its direct effects on crayfish behaviour, lighting may strongly influence periphyton growth in aquaculture rearing tanks (Wasielesky et al. 2012). Grazing represents a major foraging strategy for omnivorous crayfish (Flint and Goldman 1975; Evans-White and Lamberti 2005) and periphyton is common in the natural diet of noble crayfish (Hessen and Skurdal 1986; Westman et al. 1986). Despite several studies on the effects of lighting on crayfish performance, none of them has yet incorporated the potential benefits of associated periphyton growth as a complementary food source for juvenile astacid crayfish in intensive culture.

To enhance our knowledge on these aspects during nursery of noble crayfish, we conducted two subsequent eight-week experimental trials to investigate the effects of different light sources (fluorescent light tubes FLT, light emitting diodes LED, natural sunlight NSL) on periphyton growth and performance of juvenile noble crayfish. Two batches of artificially incubated juvenile crayfish were stocked in the nursery tanks at two different time points with temperature of process water depended on ambient conditions and elevated by a greenhouse. The start of the early trial on 28th of March represents a feasible starting point for an 8-week nursery of early-hatched juveniles while the second starting point on 21st of May is close to the time when stage II juveniles may be either reared in a nursery system or directly stocked in earthen ponds in central Europe.

Material and Methods

Two consecutive experiments (early spring trial (EST): 28th of March until 20th of May = 53 days; late spring trial (LST): 21st of May until 18th of July = 58 days) were performed with the same experimental setup and the same experimental protocol to study the effects of stocking time (EST vs. LST) on performance of juvenile crayfish during nursery. Within each trial, we set up four lighting treatments (dark control (dark), light emitting diodes (LED), fluorescent light tubes (FLT) and natural sunlight (NSL)) in triplicate to assess the effect of these lighting methods on performance of juvenile noble crayfish during nursery.

Origin of juvenile crayfish

Crayfish broodstock was obtained from earthen ponds at Oeversee Crayfish Farm, Oeversee, Germany. Two batches of crayfish eggs were artificially incubated in a hemputin incubator (Järvenpää and Ilmarinen 1990, Jones and Valverde 2020) at an eight-week interval to ensure availability of stage II juveniles for each experiment. After moulting to stage II, juveniles from each batch were carefully mixed and stocked into the experimental tanks at densities of 190 m^{-2} in EST and 160 m^{-2} in LST yielding a total of 7200 (600 per tank) and 6000 (500 per tank) stage II juveniles initially stocked in each experiment, respectively.

Experimental set-up

Nursery system

The experiments were set up in an indoor based recirculating system consisting of 20 black circular polyethylene tanks (2 m diameter and 0.8 m height, Kunststoff-Spranger GmbH, Plauen, Germany) filled with pond water. Ten tanks were exposed to natural sunlight in a greenhouse while the remaining ten tanks were placed indoors in an adjacent hall. Water was circulating between all tanks at an exchange rate of 2.5m^3*h^{-1}*$tank^{-1}$, ensuring equal water quality independent of lighting treatment in all tanks. Temperature of process water depended on ambient temperature and water was heated solely by the greenhouse. From the 20 tanks of the recirculating system, three tanks from the greenhouse and nine tanks from the hall were used for the experiment. Each experimental tank was equipped with 25 clay bricks (33 holes each) providing structure and shelter (1.4 holes/crayfish in EST and 1.7 holes/crayfish in LST).

Furthermore, a thin layer of sand as well as leaf litter (*Acer campestre*) and barley straw (*Hordeum vulgare*) were added to each tank.

Temperature, pH, dissolved oxygen and conductivity were measured twice a week in each tank during afternoon hours using a WTW Multi 350i together with a MPP350 (WTW, Weilheim, Germany). Water samples were filtered (Whatman GF/C) and analysed for nitrate (both trials all < 5 mg/L), nitrite (both trials all < 0.02 mg/L) and ammonium (both trials all < 0.07 mg/L) with continuous flow analysis (Alliance Evolution II, Alliance Instruments GmbH, Freising, Germany) and volumetric analysis was used to determine alkalinity twice a week.

Light sources

Three different lighting treatments and a dark control were set up in triplicate to assess the effect of lighting on performance of juvenile noble crayfish. One treatment was illuminated by light emitting diodes (LED), one treatment by fluorescent light tubes (FLT) and one treatment exposed to natural sunlight (NSL). For the LED treatment, a total of 27 LED (3x cold white, warm white, amber, 6x red, green and blue, Germtec GmbH & Co. KG, Herborn, Germany) were used per tank. LED lamps (IP68 standard allowing underwater installation) were immersed directly under the water surface to reduce reflection while still evenly illuminating the tanks. The FLT treatment was illuminated by one Osram Lumilux skywhite fluorescent light tube that was installed 0.3 m above the water surface. The artificial (LED, FLT) light regimes were identical in total power consumption (36 W) but differed in irradiance and spectral composition (Fig. 1). Irradiance and spectral composition were measured with a DM 150 double monochromator together with a 267 current amplifier (both Bentham Instruments Ltd. Reading, Berkshire, UK). For the artificial light regimes, measurements were performed in the middle of the tanks in the same distance from the light source were periphyton samples were obtained. The NSL spectrum was measured around noon at a light day during late spring. Lighting schedules of LED and FLT tanks were manually adjusted to the natural photoperiod twice a week. No dim light periods were included in the artificial lighting regimes. To avoid interference by scattered light each tank was covered with white plastic foil.

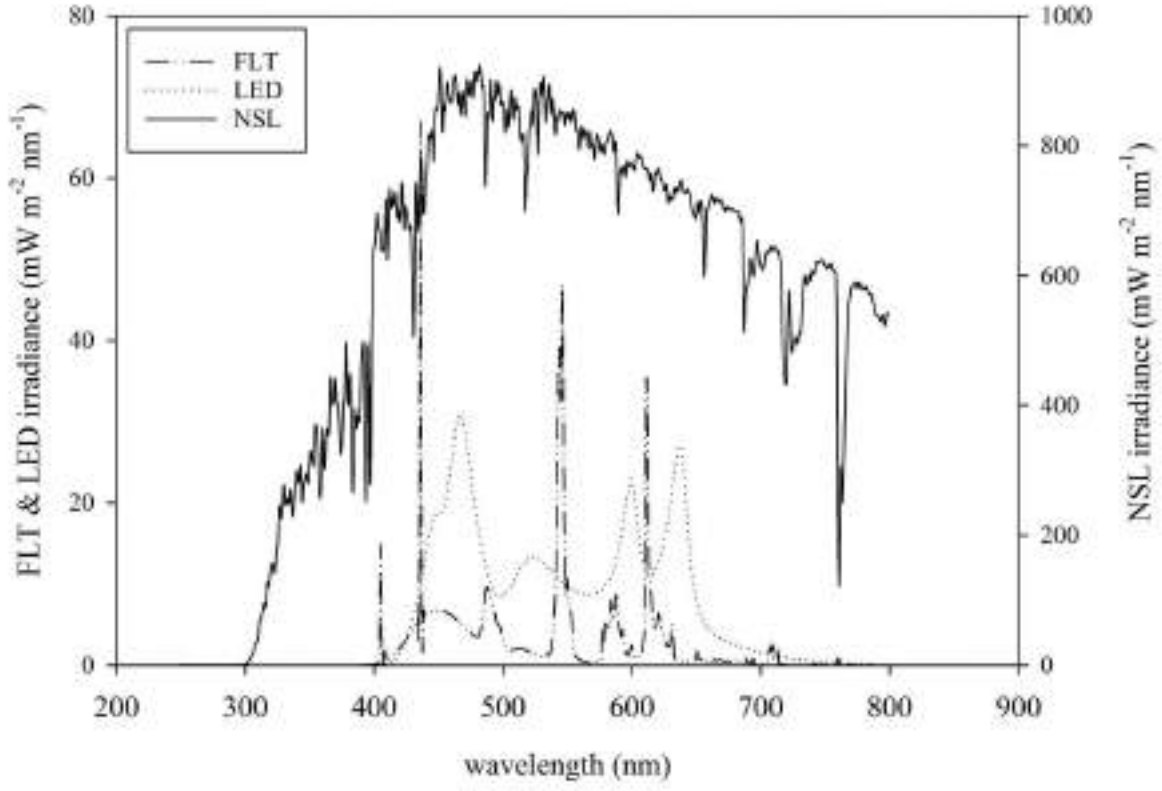

Figure 1: Emission spectra of the utilised fluorescent light tube (FLT), light emitting diodes (LED) and natural sunlight (NSL). Total photosynthetically active photon flux density between 400-700 nm were 5.4 µmol*m-2*s-2 (FLT), 15.5 µmol*m-2*s-2 (LED) and 1063.1 µmol*m-2*s-2 (NSL). Note the different scales used for the artificial light sources and NSL.

Feed and Feeding

Crayfish were fed an equal mixture of two commercial feeds (CF1: protein 53 %, lipid 15 %, carbohydrate 12 %, ash 13 %, water 7 % and CF2: protein 21 %, lipids 4 %, carbohydrates 52 %, ash 12% water 12%, both Nofima AS, Tromsø, Norway). Pellets were ground and fed at a particle size < 0.63 mm four to five times a week at a rate of 2-4 % of wet weight*day^{-1} depending on temperature and moulting stage.

Performance of juvenile crayfish

Survival was determined at the end of the experimental trials by emptying the tanks and counting all remaining crayfish. Wet weight (WW) was used to determine performance of juvenile crayfish. A subsample was weighed prior to stocking to assess initial individual weight (early trial = 34.7 ± 6.3 mg; n = 35; late trial = 39.9 ± 3.3 mg; n = 50). Halfway through and at the end of the experiment, a subsample of 50 (EST) and 30 (LST) crayfish from each replicate tank was weighed

individually. All weight measurements were performed to the nearest mg on a Kern PCB 400-1 balance. Additionally, specific growth rate (SGR) as proposed by Evans & Jussila (1997) was calculated for each replicate tank based on the means of the subsample:

$$SGR = \ln (W_f) – \ln (W_i) / t * 100$$

where W_i is the initial individual weight, W_f is the final individual weight and t is the time period of the study.

Periphyton analysis

At the end of each experiment, a quantitative periphyton sample was obtained from each replicate tank by sampling the surface of three clay bricks (total surface area 810 cm^2). Periphyton was washed off with tap water, remnants were removed with a toothbrush and the obtained suspension was transferred to the laboratory for further analysis. Examination of chlorophyll a was performed by suspending periphyton in tap water and subsequent analysis of the suspension by fluorescence spectrometry (Fluoroprobe, BBE Moldaenke, Kronshagen, Germany). This facilitated 1) quantitative assessment of chlorophyll a and 2) identification of chlorophyll a from green algae, diatoms, cyanobacteria and chryptophyta as the respective chlorophyll a molecules show a unique fluorescence spectrum. To assess dry mass (DM) and ash free dry mass (AFDM), a subsample of the suspension was filtered on pre-weighed glass fibre filters (Whatman GF/C), dried (48h, 60°C) and reweighed. Afterwards, filters were muffled (1h, 500°C) and reweighed. All periphyton weight measurements were performed to the nearest 0.001 mg on a Sartorius SC 2 microbalance (Sartorius AG, Göttingen, Germany).

Stable isotope analysis

At the end of the LST, ten individual crayfish from each replicate tank were frozen (-20°C) for analysis of stable isotope (^{13}C, ^{15}N) ratios. From the defrosted individual, the abdominal muscle was removed and dried (48h, 60°C). Dry tissue subsamples from each replicate tank were pooled (2 ± 0.05 mg). The pool sample was ground in a 2 ml safe-lock tube (Eppendorf, Germany) by adding some glass beads (Carl Roth GmbH, Germany) and homogenizing for 5 minutes (Geno/Grinder 2000, SPEX CertiPrep, Metuchen, United States). To assess variation in stable isotope signature within replicate tanks, animals from two replicate tanks were analysed individually. Samples of utilised commercial feeds and potential natural food sources (periphyton, detritus, macroinvertebrate insect larvae: Ephemeroptera: Baetidae, Caenidae, Diptera:

Chironomidae) from the tanks were dried (48h, 60°C) and homogenised as described above for crayfish tissue.

Crayfish tissue and potential food source samples were packed in tin capsules (HEKAtech GmbH, Germany). All samples were analysed for total C, total N and ^{13}C and ^{15}N at the Stable Isotope Facility of the Department of Plant Sciences, University of California (Davis, CA, USA) using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Isotopic ratios of the heavier to lighter isotope are expressed as delta (δ) values, defined as deviation from the isotopic standard reference materials (Vienna PeeDee Belemnite for ^{13}C and air for ^{15}N) and reported in ppt (‰).

Data analysis

Data were analysed using LibreOffice 6.1.5.2 and SPSS 27 for Windows. Simple linear regressions for periphyton characteristics and crayfish final wet weight were calculated using SigmaPlot 13.0. All graphs were created using SigmaPlot 13.0. Differences were considered significant at $p<0.05$. During take down of the LST, animals from a NSL replicate tank were accidentally mixed with animals from a tank that was not part of the experiment. Therefore, the respective replicate tank had to be excluded from further analysis.

Differences in crayfish performance parameters (survival, wet weight, specific growth rate) and periphyton characteristics (dry matter, ash free dry matter, chlorophyll a and chlorophyll a of green algae) were analysed using mixed-model, nested analysis of variances (ANOVA) with lighting regime nested within trial and both treated as fixed factors. Lighting regimes were chosen to represent different applicable and available solutions for indoor based recirculating aquaculture systems and the experimental trials represent two feasible starting points for nursery systems of *A. astacus* in temperate regions with (EST) and without (LST) accelerated artificial incubation of crayfish eggs. Differences of these parameters within each trial were compared using One-Way ANOVA (*p*-values based on Tukey-HSD post-hoc tests) after testing for homogeneity of variances (Levene $p>0.05$) and normal distribution of data (Shapiro-Wilk $p>0.05$). Differences in SGR between the first and second half of each trial were compared using students *t*-test (EST) or Mann-Whitney U-test (LST). Simple linear regressions were calculated for final wet weight of crayfish and periphyton characteristics (dry matter, ash free dry matter, chlorophyll a and chlorophyll a of green algae).

To validate pooling of subsamples prior to stable isotope analysis, variation of stable isotope signature within two replicate tanks was compared to variation between the respective replicate tanks of the same treatment using students *t*-test. Differences in stable isotope signature of crayfish muscle tissue ($\delta^{13}C$ and $\delta^{15}N$) within each trial were compared using One-Way ANOVA (*p*-values based on Tukey-HSD post-hoc tests). We used the MixSIR Bayesian-mixing model (Moore & Semmens 2008) to estimate feasible contributions of potential food sources to the stable isotope signature of the crayfish. Fractionation factors of the isotopic signatures of potential food sources were estimated using the method described by Caut et al. (2009), as this procedure accounts for variation in δ values of potential food sources and associated differences in isotopic fractionation.

Results

Water quality

All assessed nutrient parameters remained in a suitable range for juvenile crayfish in both trials (NO_3^- early and late all < 5 mg/L, NO_2^- early and late all < 0.02 mg/L, NH_4^+ early and late all < 0.07 mg/L, alkalinity early and late all > 4 °dH) and did not differ significantly between treatments within each trial (One-Way ANOVA Tukey-HSD all $p>0.320$). Temperature continuously increased from 12.7 to 17.8 °C in during EST (mean ± SD = 16.4 ± 2.0°C) and ranged from 18.2 to 23.7 °C in LST (mean ± SD = 21.1 ± 1.2 °C). Similar temperatures were observed in all lighting regimes at any date (One-Way ANOVA Tukey-HSD all $p>0.057$) with a tendency of NSL tanks to be up to 0.5°C elevated above LED, FLT and dark controls. Conductivity ranged from 517 to 619 µS/cm in early and from 359 to 422 µS/cm in LST. No significant differences were observed between lighting regimes at any date (all $p>0.804$). Oxygen saturation ranged between 98.0 and 112.1 % in EST and between 95.8 and 124.8 % in LST with higher saturation in NSL tanks on some occasions ($p<0.05$). No differences were observed for pH in EST (8.12 – 8.57, all $p>0.106$) but in LST pH (range 7.98 – 8.95) was frequently elevated up to 0.1 pH in NSL tanks compared to the artificial lighting regimes and the dark control ($p<0.05$). Alkalinity continuously decreased from 2.28 to 1.74 mmol/L in EST and remained between 1.81 and 1.51 mmol/L in LST (Fig.2).

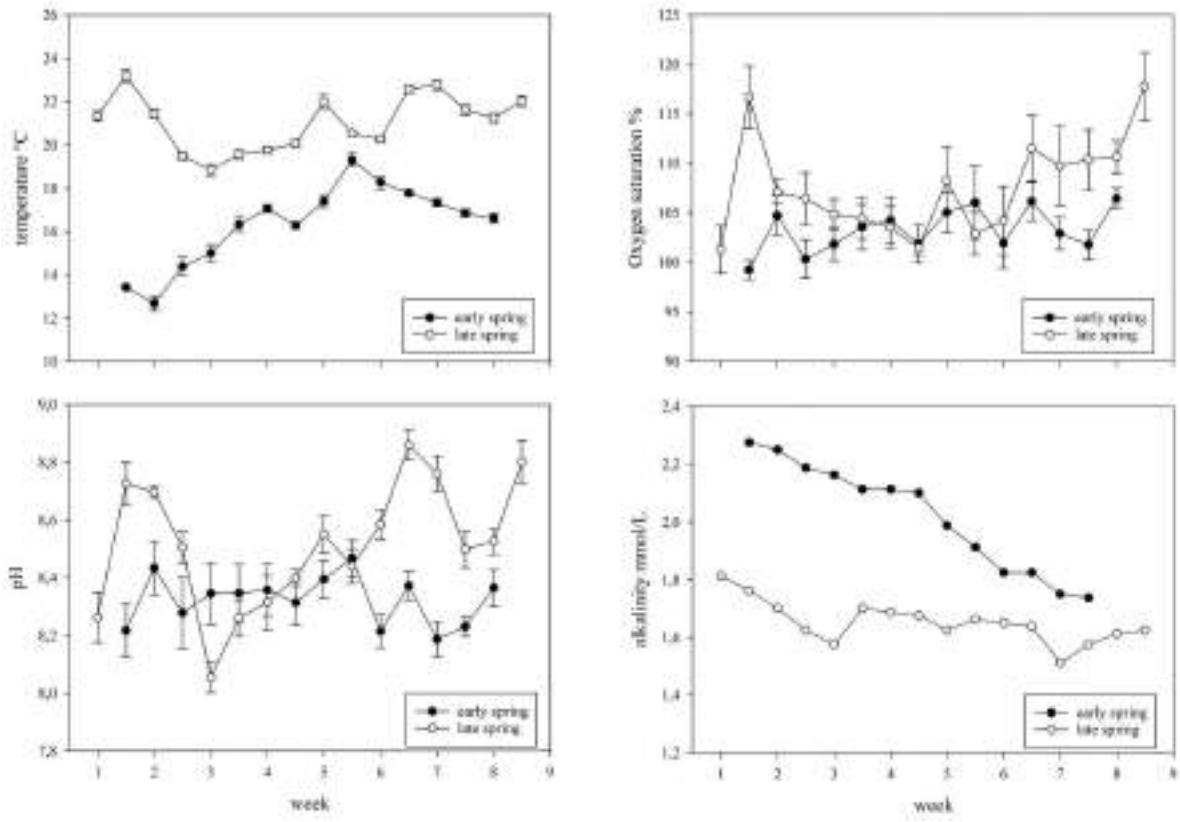

Figure 2: Bi-weekly measurements of temperature, pH, oxygen saturation in the crayfish tanks (mean ± SD, n = 12) and alkalinity (single measurements) over the experimental period in early (53 days) and late (58 days) spring during afternoon hours. In late spring, water from adjacent crayfish ponds was added to account for evaporation in week three and seven.

Crayfish performance

Table 1 summarises juvenile crayfish performance of both experimental trials. Crayfish survival was generally higher in LST than in EST (mixed ANOVA $F_{1,15}$=14.495; p=0.002) but was not equally affected by lighting regime in both trials ($F_{3,15}$=0.977; p=0.430). No differences were found between regimes in EST (One-Way ANOVA $F_{3,8}$=0.083; p=0.967), but lighting regime did affect survival in LST (One- Way ANOVA $F_{3,7}$=56.798; p=0.008) with more surviving individuals in NSL and FLT than under dark conditions (Tukey-HSD both p<0.05).

We found higher final wet weight in LST than in EST (mixed ANOVA $F_{1,15}$=441.727; p<0.001) with animals multiplying their initial wet weight about threefold in EST and tenfold in LST. There was as well a consistent and clear effect of lighting regime nested within trial on final wet weight (mixed ANOVA $F_{3,15}$=7.750; p=0.002) with NSL constantly yielding highest individual wet weight at the end of both trials (Tukey-HSD all p<0.05) and no significant differences between the remaining three lighting regimes (all p>0.652).

Table 1: Performance of juvenile noble crayfish after rearing for 53 (EST) or 58 days (LST) under different light sources. Wet weight and SGR are shown for the first (1st) and second half (2nd) half of the trials. Within each trial, values in a row without a letter in common are significantly different (Tukey-HSD p<0.05). EST = early spring trial; LST = late spring trial; dark = dark control; LED = light emitting diodes; FLT = fluorescent light tube; NSL = natural sunlight; SGR = specific growth rate; tot. = total experimental duration.

		EST				LST			
		dark	LED	FLT	NSL	dark	LED	FLT	NSL
Wet weight [mg]	1st	49.5 ± 5.5^{a}	52.0 ± 0.8^{a}	55.5 ± 3.0^{ab}	61.7 ± 3.1^{b}	123.3 ± 8.2^{a}	126.5 ± 1.9^{a}	126.0 ± 2.5^{a}	124.7 ± 6.4^{a}
	2nd	83.4 ± 13.6^{a}	83.0 ± 5.8^{a}	88.3 ± 2.5^{a}	121.9 ± 4.9^{b}	325.4 ± 51.1^{a}	349.2 ± 56.9^{a}	373.8 ± 44.6^{a}	553.6 ± 42.7^{b}
SGR [%/day]	1st	1.20 ± 0.37^{a}	1.38 ± 0.05^{ab}	1.59 ± 0.18^{ab}	1.95 ± 0.17^{b}	4.02 ± 0.23^{a}	4.12 ± 0.05^{a}	4.10 ± 0.07^{a}	4.17 ± 0.05^{a}
	2nd	2.02 ± 0.64^{a}	2.02 ± 0.30^{a}	2.18 ± 0.35^{a}	2.96 ± 0.17^{a}	3.21 ± 0.54^{a}	3.36 ± 0.55^{a}	3.61 ± 0.39^{ab}	4.97 ± 0.26^{b}
	tot.	1.65 ± 0.32^{a}	1.66 ± 0.13^{a}	1.78 ± 0.05^{a}	2.39 ± 0.08^{b}	3.60 ± 0.28^{a}	3.72 ± 0.28^{a}	3.85 ± 0.20^{ab}	4.53 ± 0.13^{b}
Survival [%]	tot.	77.4 ± 7.7^{a}	77.2 ± 0.2^{a}	78.7 ± 5.1^{a}	75.6 ± 12.0^{a}	80.8 ± 3.7^{a}	84.8 ± 1.7^{ab}	89.9 ± 1.0^{b}	90.4 ± 2.8^{b}
Biomass [g/m²]	tot.	10.3 ± 2.0^{a}	10.2 ± 0.7^{a}	11.0 ± 0.4^{ab}	14.6 ± 1.8^{b}	50.2 ± 8.4^{a}	56.7 ± 10.2^{a}	64.3 ± 8.3^{a}	95.5 ± 4.4^{b}

Specific growth rate over the whole experimental period was higher in LST than in EST (mixed ANOVA $F_{1,15}$=529.412; p<0.001) but was not equally affected by lighting regime in both trials ($F_{3,15}$=0.201; p=0.894). In both trials, specific growth rates over the entire period were significantly higher under NSL than in all other groups (Tukey-HSD all p<0.05) with the exception of FLT in LST (Tukey-HSD p=0.068). No significant differences in growth rates were observed at any date between dark, LED and FLT lighting (Tukey-HSD all p>0.638). In EST, SGR was lower during the first half of the experiment than during the second half (t-test p<0.001). The opposite was observed in LST, where SGR was higher during the second half than during the first half (Mann-Whitney U-test p<0.05).

Periphyton

We observed periphyton growth in both trials and in all treatments with NSL generating highest periphyton growth and similar mean values in both trials (Fig. 3). Differences between trials were found for chlorophyll a of green algae (mixed $F_{1,15}$=11.764; p=0.004). Lighting regime clearly affected all assessed parameters (mixed all $F_{3,15}$>12.689 all p<0.001). Lighting regime nested

within trial was not significant for any assessed parameter (mixed all $F_{3,15}$<0.832 all p>0.497) indicating different effects of lighting in the different trials.

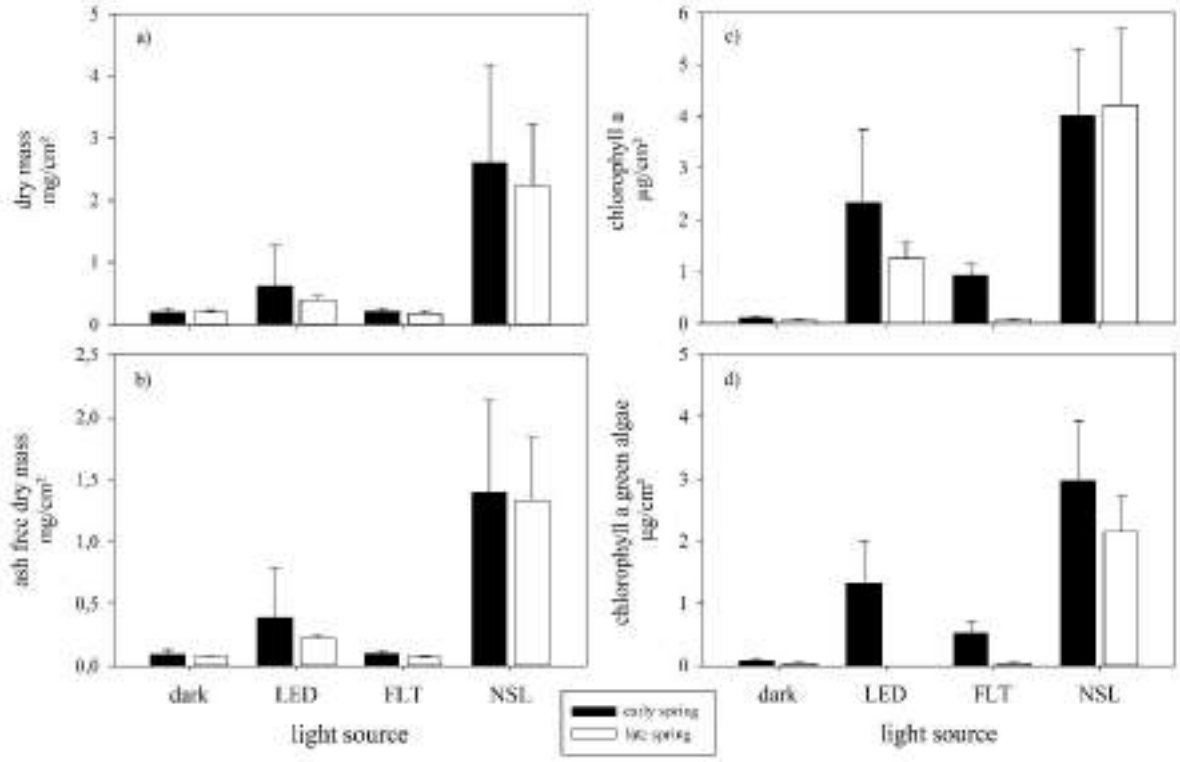

Figure 3: Periphyton dry mass (DM), ash-free dry mass (AFDM), chlorophyll a and chlorophyll a of green algae in response to different light sources in the crayfish tanks (mean ± SD, n = 3). Samples were obtained from artificial clay substrates at the end of each experimental trial. NSL yielded highest periphyton growth in both trials regarding all assessed parameters. Chlorophyll a and chlorophyll a of green algae were reduced in late spring under FLT. No green algae were detected in late spring under LED. dark = dark control; LED = light emitting diodes; FLT = fluorescent light tube; NSL = natural sunlight.

Namely, total chlorophyll a and chlorophyll a of green algae were reduced in LST under FLT lighting while LED lighting in LST did not support growth of green algae (Fig. 4). A positive correlation of periphyton dry matter and crayfish individual wet weight was observed in EST (y=15.71x + 80.01; R^2=0.93; p=0.035) and LST (y=101.85x + 324.41; R^2=0.95; p=0.026). Mean individual wet weight was positively correlated with chlorophyll a of green algae in LST (y=96.51x + 346.98; R^2=0.96; p=0.018) while total chlorophyll a was not significant in both trials.

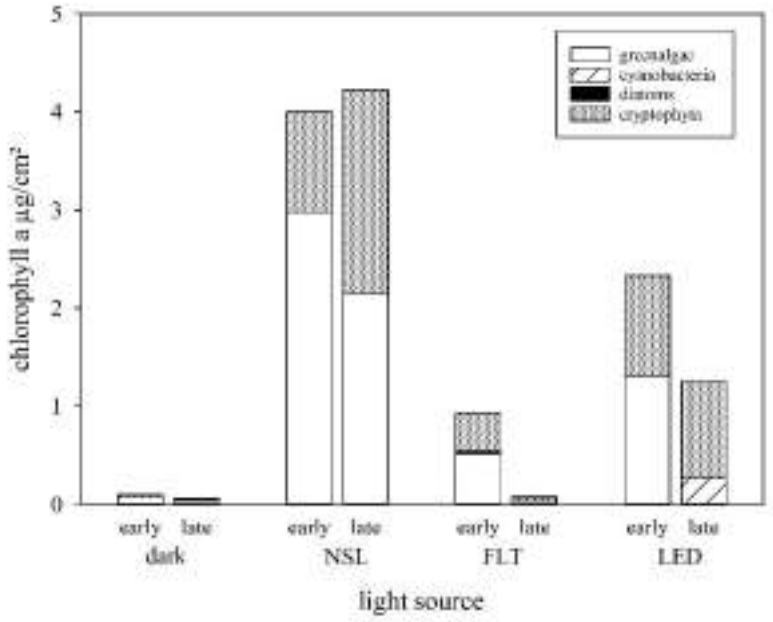

Figure 4: Algae classes that grew under different light sources in early spring (early) and late spring (late) experimental trial. Groups were quantitatively separated by fluorescence spectrometry. Only green algae and chryptophyta were detected in dark and NSL without compositional changes between trials. A small proportion (2.3 ± 2.1 %) of diatoms were detected in FLT tanks in early spring, but those did not appear in the late spring trial. Major changes in algae composition between trials were observed in LED tanks where green algae were found in early spring but completely replaced by cyanobacteria in late spring. dark = dark control; LED = light emitting diodes; FLT = fluorescent light tube; NSL = natural sunlight.

Stable isotopes

Individually analysed crayfish muscle tissue from two tanks showed little variation in isotopic signatures (dark: $\delta^{13}C$ = -21.10 ± 0.12, $\delta^{15}N$ = 10.42 ± 0.20; NSL: $\delta^{13}C$ = -20.82 ± 0.18 $\delta^{15}N$ = 9.24 ± 0.18; all n = 10) indicating that the animals in each tank were in equilibrium with their diet (Carolan et al. 2012) and using similar food sources (Sweeting et al. 2005). Variation within tanks was similar to variation between replicates of the respective treatment (*t*-test, all t_2<2.041, p>0.178) validating our method of pooling samples from ten individuals prior to stable isotope analysis.

Isotopic signatures of crayfish and potential food sources are shown in Fig. 5 before (a) and after (b) correction for trophic fractionation. Significant differences in $\delta^{13}C$ and $\delta^{15}N$ between lighting regimes were observed for $\delta^{13}C$ (One-Way ANOVA $F_{3,7}$=8.781, Tukey-HSD all p<0.05) and ^{15}N (One-Way ANOVA $F_{3,7}$=36.387, Tukey-HSD all p<0.001) between NSL and the remaining regimes. No differences were observed for $\delta^{13}C$ and $\delta^{15}N$ between FLT, LED and the dark control ($\delta^{13}C$: all p>0.856; $\delta^{15}N$ all p>0.286).

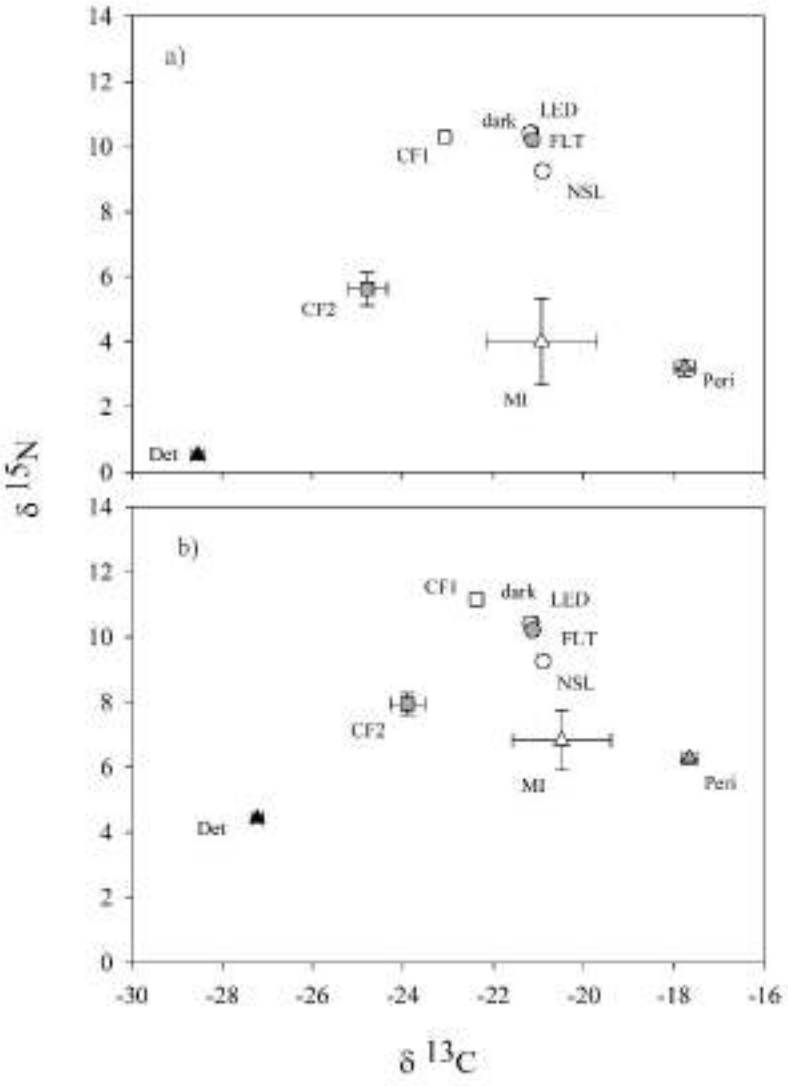

Figure 5: Dual-isotope plot on mean δ ^{15}N and δ ^{13}C ± SD of crayfish muscle tissue and potential food sources in late spring before (a) and after (b) correction for trophic fractionation. The method suggested by Caut et al. (2009) was applied to calculate the discrimination factor. Circles indicate stable isotope signature of crayfish muscle tissue, squares indicate commercial feeds and triangles indicate naturally occurring food items. CF1 = high protein crayfish feed, CF2 = low protein crayfish feed, Det = detritus, MI = macroinvertebrates, Peri = periphyton.

Consequently, the respective δ values were pooled for further analysis of the estimated contribution of potential food sources to crayfish nutrition with the mixing model. Five potential food sources (CF1 high protein feed, CF2 low protein feed, Peri = periphyton, Det = detritus, MI = macroinvertebrate insects) were identified based on qualitative samples from the experimental tanks and used in the mixing model. The high protein feed CF1 contributed most to the stable isotope signature of crayfish muscle tissue while low protein feed CF2 and detritus were of minor importance in both trials. Differences in estimated assimilated food sources between trials were observed for periphyton and macroinvertebrate insects (Fig. 6).

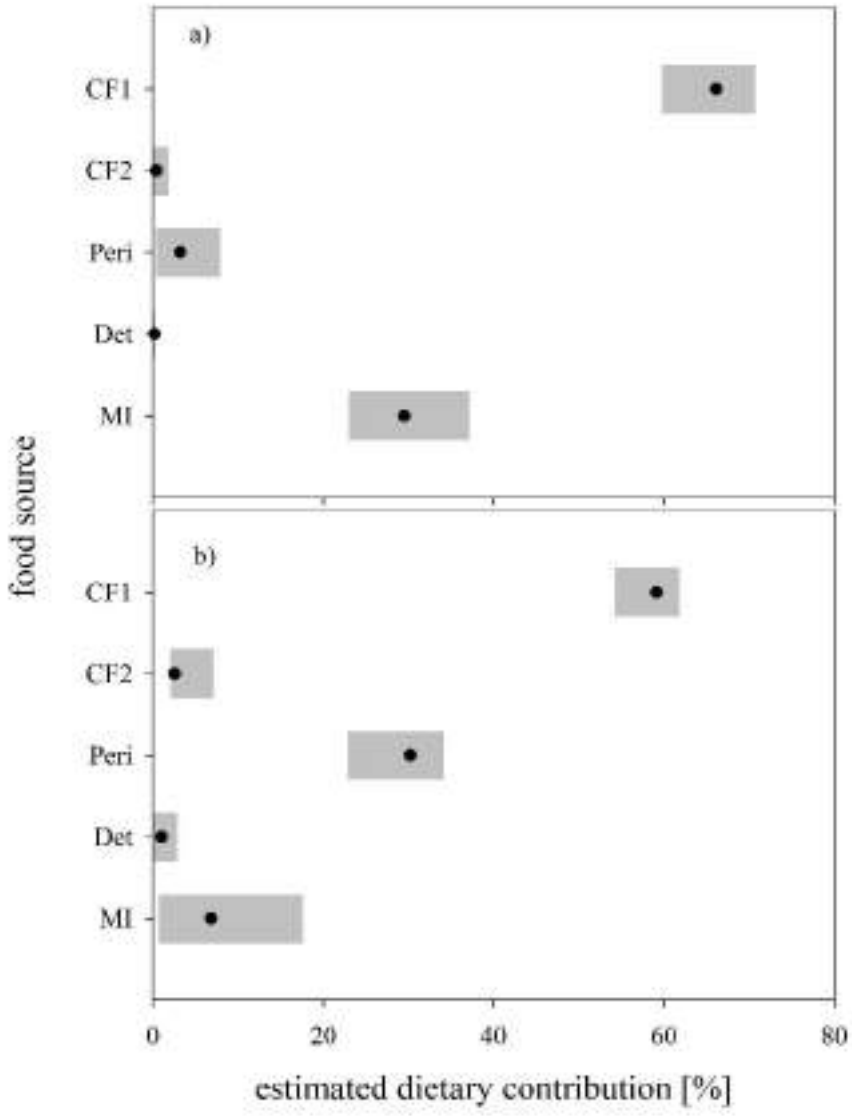

Figure 6: Estimated dietary contributions of potential food sources to the dual-isotopic signatures (δ 15N, δ 13C) of crayfish abdominal muscle tissue in late spring for pooled artificial light sources and dark control (a) and natural sunlight NSL (b). Horizontal bars show the 5 to 95 percentile range of feasible contributions, black dots indicate median. CF1 = high protein crayfish feed, CF2 = low protein crayfish feed, Det = detritus, MI = macroinvertebrate insects, Peri = periphyton.

Discussion

Trial and temperature

In large-scale commercial nursery of crayfish, temperature can only be controlled to some extent and is dependent on ambient temperature (Pursiainen et al. 1983; Garca de Yta 2016). We used a greenhouse without additional heating to generate suitable temperature conditions for juvenile crayfish already in early spring and all assessed water quality parameters remained in an acceptable range for crayfish in both trials (Fig. 2). At the beginning of the early spring trial (EST), water temperature was 13.4 °C and constantly remained well above the minimum temperature of 10 °C required for moulting of noble crayfish (Henttonen et al. 1993). Optimal temperature for growth of noble crayfish is, however, between 16-24 °C (Jussila 1997; Füreder 2016) and within this range, the effect of higher water temperature on growth of astacid crayfish is well known (Lowery 1988; Reynolds 2002). In agreement with previous studies, we observed higher growth rates at higher water temperatures in late spring trial (LST). Higher growth at higher temperatures in juvenile astacid crayfish is predominantly caused by increased moulting frequency (Kozák et al. 2009; Kouba et al. 2010) which, in turn, enhances the animals' risk to die during moulting.

Therefore, higher growth at elevated temperatures is sometimes accompanied by lower survival in astacid crayfish (Kouba et al. 2010) which was not detected in our study. An explanation for the lower survival in EST may be death of animals due to a temperature shock when transferred from the incubator (18.5 °C) to the rearing tanks (13.4 °C). However, Kozák and Policar (2003) found juvenile *A. astacus* to be resistant towards short term temperature shocks and temperature gradients as in our study can be considered to be of minor relevance. Moreover, earlier hatched crayfish for the EST were subjected to a shorter diapause during incubation than their late hatched conspecifics. In accordance with the findings of Lehmann et al. (2021), stage II crayfish that hatched for the EST had a lower weight than those hatched eight weeks later for the LST. A shortened diapause may lead to the exhaustion of metabolic reserves (Reynolds 2002; García-Guerrero et al. 2003) with potential carry-over effects from embryo to early juvenile stages (Ituarte et al. 2019). However, Lehmann et al. (2021) did not find such negative effects if accelerated hatching occurred from late February to mid-march and it is therefore unlikely that earlier hatching caused the low survival rates observed in EST. Instead, lower survival at lower temperatures in EST is in accordance with Policar et al. (2010) who reported low survival for *A. pallipes* juveniles reared at 13.3 ± 0.8 °C.

Together with the lower growth rates during the EST, the low survival illustrates that temperatures in EST, in particular during the first half of the experiment, were below optimum temperature for rearing juvenile noble crayfish. This conclusion is supported by the increase of specific growth rates over the course of the EST despite the general pattern, that growth rate in crayfish decreases with age as a result of prolonged intermoult periods (Kozák et al. 2009; Kouba et al. 2010) which we observed in LST. These high growth and survival rates obtained in LST indicate suitable temperature conditions for the animals. A further increase in water temperature in EST may be promoted by exposing relatively more tanks to direct sunlight in the greenhouse.

Lighting and agnostic interactions

Mortality caused by agnostic interactions of conspecifics is a major challenge in crayfish culture (Romano and Zeng 2017), in particular at higher densities that are required for an economically feasible production (Mazlum 2007; Ramalo et al. 2008; González et al. 2011). Most crayfish exhibit a nocturnal activity pattern (Page and Larimer 1972; Bojsen et al. 1998; Barbaresi and Gherardi 2001; Miranda-Anaya 2004) and a distinct light-dark cycle favours higher survival by synchronizing moulting to daytime hours (Franke et al. 2013) or by diminishing the animals' activity (Westin and Gydemo 1988; Taugbøl and Skurdal 1992). In our study, effects of lighting on survival were only apparent in LST at higher temperature when the animals showed higher growth rates than in EST. This is reasonable as crayfish are highly vulnerable to cannibalism after moulting and therefore, negative effects of agnostic interactions are more likely to affect survival at higher growth rates as these are accompanied by increased moulting frequencies (Kouba et al. 2010). The positive effect of NSL and FLT lighting on survival is contrary to the results from González et al. (2011) who did not observe a positive effect of a natural photoperiod on early juveniles of *P. leniusculus*. *A. astacus* is, however, more strictly nocturnal than *P. leniusculus* (Westin and Gydemo 1988; Styrishave et al. 2007) which may explain the observed differences between the two species. Furthermore, we did not observe a consistent effect of light intensity on crayfish growth or survival as positive effects were either found under highest (NSL) or lowest (FLT) irradiance (Fig. 1, Tab. 1). Low natural light intensities (<800 lux) have proven sufficient to entrain a clear nocturnal activity pattern in juvenile astacid crayfish (Westin and Gydemo 1988; Nyström 1994) and higher growth was as well observed at low artificial light intensity (38 lux) for noble crayfish summerlings (Abeel et al. 2016). Consequently, other indirect effects of lighting may have influenced the performance of crayfish in our study.

Natural occurring food items as a complementary food source

Stable isotope analysis and the results of the mixing model indicate that the high protein crayfish feed contributed most to crayfish growth regardless of lighting conditions. This is in accordance with previous results (Carral et al. 2011; Fuertes et al. 2013a; 2013b) that juvenile astacid crayfish require feeds with a high protein content. Consequently, low protein feed and detritus (Parkyn and Collier 2002) were of minor importance. Other naturally occurring food items served as important complementary food sources with differences in the significance of macroinvertebrate insect larvae and periphyton between NSL and the remaining groups. It is widely accepted that natural food items positively affect crayfish performance under culture conditions due to the lack of a suitable commercial diet (Pursiainen et al. 1983; Sáez-Royuela et al. 2007; González et al. 2008; Duffy et al. 2011). For juvenile crayfish, zooplankton has often been suggested as a suitable food item from the onset of exogenous feeding (Brown et al. 1992; Jones 1995b; Verhoef et al. 1998; Sáez-Royuela et al. 2007; González et al. 2008) and macroinvertebrate insect larvae also generate good survival and growth in juveniles (Verhoef et al. 1998; Parkyn and Collier 2002).

However, crayfish are omnivorous consumers (Nyström 2002) and a reduction of periphyton has been shown in laboratory (Bicking 2013) and field studies (Flint and Goldman 1975; Phillips et al. 2009). Noble crayfish feed on periphyton (Hessen and Skurdal 1986; Westman et al. 1986) but crayfish solely dependent on this food source usually do not perform well (Brown et al. 1992; Viau et al. 2012; Bicking 2013). We observed best overall crayfish performance under NSL and this light source constantly yielded highest periphyton growth. The high contribution of periphyton to the biomass gain under NSL and the significant correlation between periphyton characteristics and final wet weight of crayfish demonstrate the importance of this food source for juvenile noble crayfish in our study. Recently, Jin et al. (2019a) suggest a reduced feeding level and an enhancement of naturally occurring food items in culture of *P. clarkii* and our study illustrates that this approach is as well promising in nursery of noble crayfish. Apparently, periphyton growth induced by the artificial light sources was not sufficient to positively affect growth of juvenile crayfish which is supported by the stable isotope signatures of crayfish muscle tissue as they did not differ from those in the dark control. A notable effect was observed under FLT where almost no chlorophyll a was detectable in LST. This reduction in the LST compared to the EST likely was caused by increased grazing activities by the now larger crayfish juveniles as a preference for diatoms and green algae within periphyton was also reported by Phillips et al. (2009).

The positive effect of a distinct light/dark cycle on survival that we detected in LST under NSL and FLT lighting was not observed under LED lighting. A possible explanation is a reverse effect of the presence of cyanobacteria that replaced green algae under LED lighting in LST. Cyanobacteria produce a wide range of secondary metabolites that are harmful to a wide range of organisms (Brownik 2016). Crayfish are known to feed on cyanobacteria and microcystins have been shown to accumulate in different tissues (Lirås et al. 1998; Wood et al. 2012; Samdal et al. 2020) with toxic effects for crayfish (An et al. 2015). Harmful effects have, however, not yet been observed under natural conditions for adult signal crayfish (Lirås et al. 1998) and juveniles of *P. clarkii* were tolerant to toxic strains of *Microcystis aeruginosa* (Vasconcelos et al. 2001). Although crayfish show some resistance to cyanotoxins (Brownik 2016), for the majority of substances the effects on early juveniles are not known.

Conclusion

Heating of process water in nursery systems of cold-water astacid crayfish is a promising approach of cost-effectively enhancing the animals' growth period in the first growing season. The lack of a suitable crayfish diet may be partly resolved by promoting periphyton growth in rearing tanks. The clear positive effect of natural sunlight on all assessed performance parameters should support the search for technical solutions to use natural sunlight for aquaculture purpose.

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GENERAL DISCUSSION

GENERAL DISCUSSION

The approach followed in this thesis is based on the study of species-specific life-cycle, behavioural and foraging characteristics. Despite some aspects important have been studied in cold-water astacid crayfish, it became clear that various details important for an intensified culture of noble crayfish are not yet fully understood.

The close cooperation between the Limnology group and Krebszucht Oeversee allowed the identification of parameters crucial for a successful implementation of a nursery phase thereby enhancing economic viability of noble crayfish aquaculture and providing novel approaches that can be applied in conservation of natural stocks. Beside the studies included in this thesis (Chapter I-III), other aspects have been subject to investigation during the 3 years of the project and resulted in successful BSc and MSc thesis that are partly considered in this general discussion.

Several studies from Ireland (e.g. Matthews and Reynolds 1995), Spain (e.g. Carral et al. 1992) and Czech Republic (e.g. Policar et al. 2004) on incubation of eggs and rearing of early juveniles used *Austropotamobius pallipes* and *Pacifastacus leniusculus* as model species. Recently, toxicological studies with *Astacus astacus* and *Procambarus virginals* as model species demonstrated the constraints of transferring results obtained from one species to another as chronic exposure resulted in similar (Laurenz et al. 2020c) or species-specific response (Laurenz et al. 2020a, 2020b). Furthermore, differences between cold-water astacid crayfish exist with respect to the degree of nocturnal behaviour (Westin and Gydemo 1988; Lozán 2000; Styrishave et al. 2007; Gonzáles et al. 2011; Franke 2013), their aggression behaviour (Söderbäck 1991; Westman et al. 2002) and important life-cycle characteristics (Reynolds et al. 1992; Reynolds 2002).

Embryonic development and accelerated artificial incubation of crayfish eggs

Under natural conditions all European astacid crayfish mate and spawn during autumn and females berry their eggs for several months until the juvenile crayfish hatch (Reynolds 2002). During the winter months at low ambient temperatures embryonic development is arrested.

There is evidence for *A. astacus* (Cukerzis et al. 1978), *A. pallipes* (Rhodes 1981) and *P. leniusculus* (Celada et al. 1988; Carral et al. 1992) that this resting period is important for successful embryonic development. In particular, species specific requirements for optimal temperature, duration of and time point for the required cold period (CP) during embryogenesis seem to exist but generalizations can be drawn if the animals' life cycle under natural conditions is taken into account.

Applicability of the Hemputin incubator

Various devices have been evaluated for the artificial incubation of crayfish eggs. Strempel (1973), Cukerzis (1973; 1988) and Cuzerkis et al. (1979) suggest the incubation in modified zug-jars. Despite their general feasibility, incubation of crayfish eggs in zug-jars has limitation for culture application. Crayfish eggs are attached to one another and to the female by an egg stalk (Thomas 1991). This results in clumping of eggs during artificial incubation leading to the collection of air bubbles and subsequent flushing of egg clutches from the zug-jars (Jeske 2007). Rhodes (1981) developed a modified flow-through technique with enhanced bottom surface area where the eggs are borne on submerged nylon mesh trays. This approach was further improved by Carral et al. (1992) by covering the upper part with an additional mesh to avoid flushing of the eggs. Similar devices have frequently been used in studies on the embryonic development of astacid crayfish eggs (e.g. Policar et al. 2006; Seemann et al. 2014). However, those devices can only hold a low number of eggs, their operation is very labour intensive and therefore their applicability for culture purpose is limited (Jeske 2007). A modified incubation method on a moving tray that was first presented by Järvenpää and Ilmarinen (1990), described in detail by Jeske (2007) and recently modified for redclaw crayfish (Jones and Valverde 2020) was used in chapter I. The results presented in chapter I are consistent with the high incubation efficiencies achieved by Jeske (2007) and the hemputin incubator likely is applicable for incubation of other European crayfish species such as *A. pallipes* of *A. torrentium*.

Embryonic diapause and significance of a cold period during embryogenesis of astacid crayfish

Accelerated artificial and maternal incubation of crayfish eggs has received considerable attention and the effect of temperature regime is of greatest interest to researchers working with K-selected crayfish species. However, most studies on accelerated incubation focus on signal crayfish *P. leniusculus* (e.g. Carral et al. 1992; González et al. 2009a) and white clawed crayfish *A.*

pallipes (e.g. Pérez et al. 1998a; 1998b; 1999; Policar et al. 2009). The information available for noble crayfish is largely based on field observations (Taugbøl and Skurdal 1995) with only little experimental evidence for an optimal temperature regime (Policar et al. 2004) and is insufficient to support a viable application of accelerated artificial incubation in culture.

The results in chapter I of this thesis show that eggs of *A. astacus* can be stripped one month after the last mating is observed in mid-December and artificial incubation can cover 95 % of total embryonic development from gastrulation to hatching. Likewise, artificial incubation can cover approximately 90 % of the total number of degree days required for incubation, respectively. This is an even longer period than reported by Pérez et al. (1998b) for *A. pallipes* and illustrates the potential of artificial incubation for noble crayfish culture if a suitable temperature regime is applied. The time period of broodstock holding is reduced and consequently, space and energy requirements are lowered. Furthermore, egg losses due to female mortality or handling of females are equally reduced (Pérez et al. 1998b; Jones and Valverde 2020).

It is well known that embryonic development of astacid crayfish can be significantly shortened by elevated temperature (e.g. Mason 1977b; Carral et al. 1992; Reynolds 1992; 2002). Westin and Gydemo (1986) obtained hatchlings of noble crayfish in the middle of February while Seemann et al. (2014) achieved hatching of the same species when incubated at 18 °C at the beginning of March. Policar et al. (2004) used a lower final incubation temperature of 12.5 ± 3 °C and hatching occurred at the beginning of April. However, Westin and Gydemo (1986) did not report relative survival and Policar et al. (2004) report lower survival in accelerated incubated eggs than those incubated maternally under natural conditions despite the inclusion of a CP of 45 days at 5.5 ± 2 °C. Seemann et al. (2014) achieved early hatching only at the expense of low survival (11 – 27 %). In the latter case, low survival was likely caused by an insufficient CP of 2 weeks at 4 °C (Seemann et al. 2014) that was even shorter than the shortest incubation period in chapter I. The inclusion of a 24- and 38-day extended CP at 7.5 ± 0.5 °C with subsequent incubation at 17.5 ± 0.4 °C in chapter I favoured hatching between beginning and the middle of March while stage II juveniles were obtained from the middle to the end of March with reasonable survival rates (50.8 ± 14.4 % and 60.0 ± 7.0 %), respectively. A further reduction of incubation period apparently is possible but the benefits for culture are questionable as negative effects on survival increase with decreasing incubation period at elevated temperatures.

Detailed information on different temperature regimes during embryonic development is available for *A. pallipes*. This species shows similar reproduction patterns to *A. astacus* under natural conditions as mating and spawning likewise occur at ambient water temperatures 10-12

°C (Reynolds et al. 1992; Grandjean et al. 2000). Rhodes (1981) studied the effect of stripping time on the hatching success during artificial incubation at different temperatures (18°C, 13°C, 11°C) when eggs were obtained from a natural population at monthly intervals and increasing lateness of stripping likely corresponded with increasing CP during embryonic development. Successful hatching was observed when eggs where stripped from late January onwards with no surviving animals at earlier stripping dates and higher incubation efficiencies at later stripping time points. Policar et al. (2009) did not find a positive effect of a prolonged CP when eggs were exposed to an initial CP of five days at 6.1 ± 1.3 °C immediately after egg laying followed by the experimental CP at 4.5 ± 0.3 °C. However, their shortest CP of 45 days was still longer than the longest CP in chapter I and relative survival rates were not reported.

With respect to the optimal time point for entering diapause in *A. pallipes*, Pérez et al. (1998a) provide some useful information. The eggs where artificially incubated at constant temperature of 10 ± 1 °C and a CP (5 ± 1 °C) of two to four weeks duration initiated at embryonic stage IX (embryo with rudiments of naupliar appendages). Subsequent incubation took place at 15 ± 1 °C until hatching. A control group without a CP and temperature rise from 10 ± 1 to 15 ± 1 °C at stage XIII (embryo with strongly developed eye pigment) was also included. The authors achieved good survival (66.7 to 72.7 %) but no effect of temperature treatment was observed. The authors conclude that moderate low temperatures (10 °C) may be sufficient to achieve good survival without a period of low temperature. However, it is slightly difficult to follow this conclusion as the temperature regime during maternal incubation and before stripping was not specified. Stripping took place 77 days after spawning at stage VIII (embryo with mandibular rudiments) and it may be assumed that the embryos experienced a CP during maternal incubation before the start of the experiment.

That the conclusion by Pérez et al (1998a) may be misleading is somehow underlined by Celada et al. (2001). The inclusion of a CP at the same embryonic stage IX and similar temperature treatments (initial incubation temperature of 10 ± 1 °C, CP 5 ± 1 °C for three weeks initiated at stage IX, subsequent incubation at 15 ± 1 °C) did consistently not improve survival during maternal incubation and no significant differences between CP were detected. Temperature before the experiment was 9 ± 2 °C without a period of lower temperature. The study yielded, however, low overall survival rates (21.9 – 32.9 %) that are similar to those obtained for the shortest CP in chapter I.

For *P. leniusculus* detailed and consistent information is available on the temperature requirements during embryogenesis. Mason (1977b) used a similar approach as Rhodes (1981)

and stripped eggs at different time points with subsequent artificial incubation. Survival increased with increasing lateness of strip when eggs were incubated at low (7-10°C) and high temperature (18°C) but no such trend was apparent at 13°C. Celada et al. (1988) applied several combinations of temperature, duration of and time points for entering CP during maternal incubation. A four week CP at 5.5 °C proved sufficient to obtain high survival and no further improvement was achieved with longer CP. The optimal time point for initiating CP was phase IX (82 % survival) but initiating CP at the beginning of gastrulation likewise yielded acceptable survival (60 %). This was confirmed by Carral et al. (1992) using artificial incubation with early stripped eggs (beginning of gastrulation) and highest survival was obtained when eggs were subjected to a CP of three weeks at 5.5 °C beginning at stage IX.

To sum up, it becomes apparent that a CP of 6-7 weeks at early developmental stages around gastrulation is beneficial both for *A. astacus* and *A. pallipes* whereas a CP in advanced embryonic development does not increase survival rates in *A. pallipes*. This is congruent with their reproductive cycle in natural habitats. However, at low ambient incubation temperatures, it is possible to obtain hatchlings under controlled conditions but only at low survival rates that are similar to those achieved for *A. astacus* at the shortest CP in chapter 1. In contrast, *P. leniusculu*s requires a shorter CP to obtain high embryonic survival and the optimal time-point to enter diapause for *P. leniusculus* is at stage IX which likewise correspond to the natural reproduction cycle as eggs are laid earlier at higher temperatures.

Planning of stocking time point using degree days

When artificial accelerated incubation is applied in culture, a major advantage is the possibility for an exact planning of stocking time (Jeske 2007). To account for variation in stripping time and applied temperature regimes, knowledge on the number of degree days required for successful embryonic development is useful. Under natural conditions in Norway, noble crayfish require between 1420 and 1900 degree days for embryonic development (Hessen et al. 1987; Taugbøl and Skurdal 1990). However, several authors report an ascending total number of degree days required for embryonic development in astacid crayfish with longer incubation period at lower incubation temperatures or longer CPs, respectively (Cukerzis et al. 1978; Harlioğlu 1996, Policar et al. 2004; 2009). For noble crayfish that were either artificially or maternally incubated at elevated temperatures, Policar et al. (2004) obtained hatchlings after 1554 degree days, Hessen et al. (1987) after 1300 degree days and Seemann et al. (2014) report hatching after only 1191 degree days. The findings in this thesis are in accordance with the former studies as the number

of degree day required for completion of embryonic development ascended with longer CPs (Appendix, Tab 3). This illustrates the limitation of the degree day approach to forecast the duration of physiological phenomena that do not necessarily show a steady increase with increasing temperature (Bonhomme 2000). However, species specific information on the temperature dependence of embryonic development presented in chapter I favours accurate planning of hatching and subsequent stocking for accelerated incubated eggs of noble crayfish.

Juvenile crayfish rearing and performance

Obtaining high growth and survival rates is a major challenge in the intensive rearing of juvenile crayfish (Kouba 2011). Complex interactions of water quality, food availability and quality, stocking density, shelter availability and lighting conditions affect the animals' physiological condition, behaviour, moulting cycle and the quality and quantity of agnostic interactions thereby influencing growth and survival.

Performance of early hatched juveniles

Information on the performance of juvenile astacid crayfish obtained by accelerated incubation is scarce and inconsistent. The interpretation of published data is likewise difficult, as temperature regimes during artificial incubation differ considerably and species specific effects may interact with unsuitable experimental procedures. Policar et al. (2004) report slower growth rates for advanced juveniles of noble crayfish that hatched 88 days earlier than those from ambient outdoor temperatures. After 90 days of rearing, normally hatched juveniles had a higher wet weight (0.305 ± 0.010 g) than the advanced juveniles (0.207 ± 0.058 g). Mortality was also higher for advanced juveniles (day 90: normally hatched = 47.2 %, advanced juveniles = 26.2 %). However, growth was generally very low in the study by Policar et al. (2004). Furthermore, Policar et al. (2004) used an incubation temperature of only 12.5 ± 3 °C degrees and juveniles were subsequently reared at 20.5 ± 0.2 °C.

For marine decapods, there is evidence for anticipatory embryonic effects to environmental conditions (i.e. salinity, temperature, oxygen) experienced after hatching (Giménez 2020) and larvae of *Macrobrachium rosenbergii* showed a wider tolerance to temperature when eggs were reared at intermediate temperature (Gomez-Diaz 1987). Palaemonidae and Astacidae show important differences in their embryonic development (e.g. absence of a larval phase in Astacidae) and anticipatory embryonic effects have not been described for crayfish. A larger

difference between incubation and rearing temperature may, in addition to a short CP, have favoured deficient performance of juvenile crayfish in comparison to those that surpassed the embryogenesis at ambient temperatures. This may partly explain the difference between the observations by Policar et al. (2004) and the results presented in chapter I, where the difference between incubation and rearing temperature was only 2.6°C and no significant differences in any recorded performance parameter were observed between stage II juvenile noble crayfish originating from different incubation temperature regimes. Instead, in chapter I there was a tendency for a positive effect of a prolonged 38-day CP of (i.e. 48-day total CP) on crayfish performance which is more congruent with the results presented by Sáez-Royuela (1995) and González et al. (2009) for stage II *P. leniusculus* that artificial incubation does not negatively influence stage II juvenile performance if a suitable incubation regime is applied.

Stable isotopes and diet back calculations

Studies estimating $\Delta^{13}C$ and $\Delta^{15}N$ frequently show a significant deviation from the commonly applied enrichment of 0.4 ± 1.3 ‰ for $\delta^{13}C$ and 3.4 ± 1 ‰ for $\delta^{15}N$ between trophic levels (Post 2002). For crayfish that feed on a variety of food items the estimation of discrimination factors is particularly difficult. Necessary feeding trials need to last for several weeks even for juvenile crayfish allowing the animal to reach isotopic equilibrium with the respective diet (Carolan et al. 2012; Laurenz 2014). Crayfish also ingest and assimilate low quality food and starvation will occur during the feeding trials if such food items are solely supplied. Starvation is known to greatly influence trophic discrimination (Doi et al. 2017) possibly resulting in misleading discrimination factors with only limited applicability apart from the experimental conditions where they have been determined.

For juvenile noble crayfish, Laurenz (2014) found $\Delta^{13}C$ to range from -4.49 ‰ for animals fed on *Chara* sp. to 6.57 ‰ for animals fed solely on detritus. A comparable wide range was reported for $\Delta^{15}N$ from 1.62 ‰ for animals fed on a low protein commercial diet to 8.53 ‰ for animals fed solely on detritus. This is a much wider range than observed for *Cherax destructor* by Carolan et al. (2012) who report $\Delta^{13}C$ of -1.1 ± 0.05 ‰ and $\Delta^{15}N$ of 1.5 ± 1.0 ‰. Similarly, Glon et al. (2016) report 95 % confidence intervals for $\Delta^{13}C$ of 0.14–1.55 ‰ (bloodworm diet) and 0.86–2.35 ‰ (algae diet) while 95 % confidence intervals for $\Delta^{15}N$ were 0.32–2.11 ‰ (bloodworm diet) and 2.06–3.08 ‰ (*Orconectes rusticus*; algae diet) or 2.53–4.51 ‰ (*O. virilis*; algae diet), respectively.

Laurenz (2014) used similar food source to those applied and identified in chapter III of this thesis. Application of the Δ values from Laurenz (2014) to the $\delta^{13}C$ and $\delta^{15}N$ data of the naturally occurring food items in chapter III and subsequent estimation of the stable isotope signature of the crayfish with the mixing model (Moore and Semmens 2008) did not favour a model result. This may have either been caused by overlooking important food sources or incorrect Δ values (Phillips 2012). The experimental conditions were beneficial for identifying all available food sources, consequently, a more robust estimation of discrimination factors $\Delta^{13}C$ and $\Delta^{15}N$ following Caut et al. (2009) was applied. This method is based on a meta-analysis of the relationship between δ and Δ and generalised equations are presented that allow the estimation of diet-tissue Δ values. The negative relationship between δ and Δ (higher δ result in lower Δ) (Caut et al. 2009) was likewise found for juvenile noble crayfish (Laurenz 2014). The estimation of Δ according to Caut et al. (2009) resulted in Δ values more close to those reported by Carolan et al. (2012) and Glon et al. (2016) ($\Delta^{13}C$ 0.091 ± 0.024 for periphyton to 1.308 ± 0.016 for detritus and $\Delta^{15}N$ of 0.863 ± 0.012 for high protein crayfish feed to 3.905 ± 0.036 for detritus; Appendix). The deviation from the Δ values determined by Laurenz (2014) was particularly apparent for those feeds that resulted in low growth rates and survival (e.g. difference for detritus $\Delta^{13}C$ 5.26 ‰ and $\Delta^{15}N$ = 4.63 ‰; difference for algae $\Delta^{13}C$ 4.60 ‰ and $\Delta^{15}N$ = 0.37 ‰) hinting at potentially confounding effects of starvation on trophic discrimination. Subsequent application to the $\delta^{13}C$ and $\delta^{15}N$ data of the naturally occurring food items facilitated a coherent result of the mixing model (Chapter III, Fig. 6). Despite remaining uncertainties I consider this a valid approach to gain insights into the importance of different natural and commercial food items for juvenile crayfish.

The use of stable isotope analysis provides a more robust estimation of the contribution of a diet item to the animals' growth than gut contents analysis as overestimation of diets that have a long latency period in the gut is reduced (e.g. Bondar 2007). However, the incorporation of other methods such as fatty acid analysis may as well have provided valuable additional information on the importance of commercial feeds and naturally occurring food items in Chapter III. This has recently been shown by Seemann et al. (2017) who found that fatty acid composition of noble crayfish summerlings that were able to feed on a natural diet in ponds was more diverse than those that fed on a mix of commercial diets supplemented by *Elodea* spp. and *Mytilus edulis*. Transferring this to the composition of naturally occurring food items in the nursery system, in particular to the periphyton, may have provided valuable insights to the natural demands of the animals thereby further aiding the development of commercial feeds.

Commercial feeds and assessment of growth

Various commercial diets have been evaluated for cold water astacid crayfish (Celada et al. 1989; 1993; González et al. 2012). They generally proved insufficient and consequently, several attempts to develop a suitable commercial feed for crayfish were made particular for juvenile crayfish (Ackefors et al. 1992; Wolf 2004; Siikavuopio et al. 2010; Carral et al. 2001; Lehmann 2012; Fuertes et al. 2013a; 2013b; 2014).

Recent efforts on feed development are primarily focussed on a diet for juvenile crayfish. Based on previous results (González et al. 2008; González 2009b; González et al. 2010; González et al. 2012), Carral et al. (2011) developed a practical diet containing 15 % dried decapsulated *Artemia* cysts and achieved comparable results to the previous studied where *Artemia* was supplemented. The composition of this died was recently modified by the substitution of fishmeal by feather meal (Fuertes et al. 2013a) and poultry by-product meal (Fuertes 2013b) and both appeared feasible at moderate substitution rates of 15 % and 45 %, respectively. For juvenile noble crayfish, Lehmann (2012) reports reasonable high growth rates (2.1-2.3 %/d) and high survival (86-98 %) for early juvenile noble crayfish either fed frozen *Daphnia* or experimental high (53.1 %) protein diets containing either 5 % shrimp or 5 % king crab meal whereas a low (21 %) protein diet resulted in similar survival but lower growth rate (1.1 %/d). Those studies generally rely on the assessment of somatic growth (i.e. weight and length gain) and even though feeding trials for juvenile crayfish can be significantly shorter than for adults, they commonly last up to three months (e.g. Carral et al. 2011).

In chapter II of this thesis I examined the applicability of RNA/DNA ratio as a complementary and alternative physiological indicator for the overall growth capacity for juvenile crayfish. The suitability of this parameter for development of feeds and culture conditions has recently been confirmed for Nile tilapia *Oreochromis niloticus* juveniles (Ding et al. 2020) and postlarvae of the pearl oyster *Pinctada fucata* (Guoliang et al. 2021) all achieving high correlations ($R^2 > 95$ %) between morphometric growth parameters and RNA/DNA ratio. For summerlings of *A. astacus* (mean wet weight 2.05 ± 0.03 g) and two summer old *A. leptodactylus* (mean wet weight 30.1 ± 8.2 g) that were fed on an experimental diet based on fish faeces Roessler et al. (2020) did not detect any of the growth performance differences in RNA/DNA ratio. However, when looking at morphometric growth parameters, it becomes apparent that hardly any weight or length gain occurred during the experimental period and significant differences were only detected for relative weight gain in *A. leptodactylus*. This somehow illustrates limitations for the applicability of RNA/DNA ratio as a growth parameter in the undesired case of starving animals in all

experimental groups or unsuitable experimental conditions. That starvation of juvenile crayfish is well reflected by RNA/DNA ratio was clearly shown in chapter II and recently confirmed for the southern king crab *Lithodes santolla* by Sacristán et al. (2019).

Nevertheless, the greatest potential for nucleic acid based growth parameters such as RNA/DNA ratio with respect to aquaculture application lies in the development of feeds for summerlings and adult crayfish. Even though a year round intensive production solely based on commercial feeds has proven unprofitable for crayfish (e.g. Huner 2002; Lewis 2002; Skurdal and Taugbøl 2002; Kozák et al 2015; Garca de Yta 2015), the intensification of semi-intensive culture considerably depends on supplementary feeds for grow out. Furthermore, in a recent review Harlioğlu and Farhadi (2017) point out the lack of knowledge on factors affecting reproduction efficiency in cultured crayfish. The development of suitable feeds is identified as a crucial factor for captive breeding of crayfish and application of nucleic acid based growth parameters may aid their development.

However, it has to be stated that to date there is no established commercial diet neither for adult nor for juvenile astacid crayfish and best growth and survival rates are frequently obtained if natural food items are supplied in addition to commercial feeds (e.g. González et al. 2008). As the European crayfish production is economically small and feed development and production costly, it is unlikely that this will change in the near future. Consequently, providing naturally occurring food items seem an adequate short- to mid-term approach to overcome the deficiencies in commercial feed supply.

Naturally occurring food items and the enhancement of periphyton growth by lighting

In particular, the supplementation of a dry diet with live *Artemia* nauplii or decapsulated *Artemia* cysts has improved survival and growth rates of juvenile *P. leniusculus* from the onset of exogenous feeding (González et al. 2008; González 2009b; González et al. 2010; González et al. 2012). Furthermore, medium (40.3 %) or high (55.5 %) crude protein likewise yielded good performance (González et al. 2012) if supplemented with *Artemia* which is in accordance with Wolf (2004) for juveniles of the same species.

The experimental diets evaluated by Lehmann (2012) were also applied in the experiments in Chapter III. Growth rates in late spring trial (LST) over a similar period were, however, approximately doubled (3.6 – 4.5 %/d) under natural sunlight. In particular, periphyton contributed significantly to the animals' isotopic signature indicating its importance for biomass

build-up. The positive effect of naturally occurring food items other than *Artemia* on survival and growth of crayfish has been documented for noble crayfish (Pursiainen et al. 1983), for *Cherax quadricarinatus* (Jones 1995b; Viau et al. 2012) and *Procambarus clarkii* (Jin et al. 2019a) but little information on their importance for juvenile noble crayfish is available. The assumption, that animal food is of major importance for juvenile astacid crayfish is based on gut content analysis of summerlings (Abrahamsson 1966; Mason 1975; Bondar 2007) and feeding trials that indicate higher growth on animal diet than on macroalgae (Laurenz 2014) or periphyton (Bicking 2013). My results as well illustrate the importance of high protein feed which is in accordance with the results from González et al. (2012). However, Wolf (2004) demonstrated that, if high protein feed is solely supplied, inhibitory effects on the animals' growth may occur. In addition to inhibition of growth, loss of carapace pigmentation indicates insufficient diets (Henttonen 1993; Paglianti and Gherardi 2004; Lehmann 2012) with unknown mid-term effects. In accordance with the results in chapter III, Celada et al. (1989) attained higher growth and survival of juvenile *P. leniusculus* when fresh plant material supplemented a commercial diet.

Enhancement of periphyton growth with artificial light sources was apparently not sufficient to support similar positive effects on growth as observed under natural sunlight. In contrast, LED lighting did favour growth of cyanobacteria that likely supressed a positive effect of light induced nocturnal locomotor rhythmicity as a result of toxic effects of cyanotoxins ingested by the crayfish (e.g. Lirås et al. 1998; Samdal et al. 2020). The intensity of the LED was higher and spectral composition different from FLT with peaks in the blue (approx. 450 nm) and orange to red (approx. 590-650 nm) wavelengths. Luimstra et al. (2018) found inhibitory effects blue (450 nm) light on different species of cyanobacteria. Similar negative effects of blue light were reported by Tyystjärvi et al. (2002) whereas the green to red light spectrum were more effectively used for photosynthesis.

In this context, the spectral sensitivity of the crayfish extraretinal photoreceptors crucial for the entrainment of circadian locomotor activity (Sullivan et al. 2009; Rodríguez-Sosa et al. 2012; Sánchez-Hernández et al. 2018) needs to be considered. At least the caudal photoreceptor has at least two photosensitive pigments apparently more sensitive to blue and green than to red light (Sánchez-Hernández et al. 2018). The nocturnal activity pattern of juvenile noble crayfish can be well entrained under blue light at moderate illuminance of 1800 lux (Häberle 2013). The entrainment of a nocturnal activity pattern has frequently proven to enhance survival and growth of crayfish under culture conditions (e.g. Mason 1978) by influencing agnostic interactions (Farca Luna et al. 2009) and synchronisation of moulting during daytime hours (Franke et al. 2013).

Therefore, the application of light sources with a higher irradiance in the blue and green spectrum may suppress growth of cyanobacteria while still facilitating the entrainment of circadian rhythms of the animals at least in juvenile noble crayfish.

Conclusions and outlook

It can be concluded that noble crayfish have the plasticity for a great shortening of embryonic development. Accelerated artificial incubation of noble crayfish eggs is feasible at high incubation efficiency and without negative effects on juvenile performance a minimum diapause period of 6 weeks at 4-7 °C at the time around gastrulation is applied. This favours hatching during mid-March and independent stage II juvenile crayfish availability towards the end of March.

At this time point, optimal rearing conditions for early hatched juvenile can be promoted by elevating the water temperature in a fully recirculating aquaculture system if the rearing tanks are partly exposed to natural sunlight in a greenhouse. However, further heating of process water may be necessary to achieve optimal water temperature for the animals. If stocked at low densities (i.e. < 200 crayfish / m^2) and supplied with a high protein diet, growth and survival rates are enhanced by lighting conditions that entrain the animals' locomotor activity and moulting patterns while simultaneously promoting periphyton growth. In that case, it is achievable to stock advanced juvenile crayfish into earthen ponds with a mean weight of 0.5 g that is otherwise reached towards the end of the growing season (Appendix, Tab. 4). In addition to a longer growing period in the first season, the size of juveniles will reduce mortality by invertebrate and fish predation (Pursiainen et al. 1983; Jeske 2007).

In particular, stocking advanced juveniles favours the production of table-size crayfish (70-100 g) within two summers. This may greatly enhance the economic viability of noble crayfish culture. Not only is the production time reduced by 30 % but with regard to the management of crayfish culture facilities another benefit arises. A majority of crayfish that underwent accelerated incubation and nursery will reach maturity within two summers and therefore, the need for separate broodstock holding facilities is reduced.

The current lack of a suitable commercial diet for juvenile astacid crayfish may be overcome by enabling the animals to feed on naturally occurring food items, in particular macroinvertebrate insect larvae and periphyton. However, the results may likewise encourage the development of commercial feeds for juvenile astacid crayfish. Aside of *Artemia*, naturally occurring food items that are part of the juvenile crayfish's diet under natural conditions should

be considered with regard to their specific contribution to the animals' nutrition and this may give insights into species specific requirements (e.g. Seemann et al. 2017). The development of such feeds for juvenile and adult crayfish may benefit from the application of nucleic acid based growth parameters.

For conservation of endangered noble crayfish in Schleswig-Holstein, ongoing projects are already applying the results obtained in this thesis. Adult crayfish caught from natural stocks reproduce in captivity and eggs are subsequently stripped and incubated according the regime presented in chapter I followed by a nursery as described in chapter III. The early hatched juveniles can be easily reared in the nursery system until stocking into gravel pit waters in autumn. They reach sexual maturity in the year following stocking and reproduction has been documented two years after hatching (Lehmann 2021).

The presented conditions for artificial incubation of crayfish eggs and subsequent nursery may be applicable to other highly endangered species of astacid crayfish with similar life cycles such as stone crayfish *A. torrentium* (Maguire et al. 2002; Dakič and Maguire 2016) and white-clawed crayfish *A. pallipes* (Reynolds et al. 1992). Similar to noble crayfish, populations of these two species are under great threat by the spread of *P. leniusculus* and crayfish plague to upstream watercourses (Chucholl and Schrimpf 2016). Species conservation programs are currently established and Berger et al. (2018) and Lovrenčič et al. (2020) highlight the importance of considering genetic information for restocking programs. Knowledge on efficient juvenile production is crucial for their success and may be of even greater importance for *A. torrentium* and *A. pallipes* due their lower fecundity (Reynolds et al. 1992; Huber and Schubart 2005; Sáez-Royuela 2006; Policar et al. 2009; Hubenova et al. 2010). Furthermore, there is upcoming evidence for species (Jussila et al. 2017) and population specific resistance and vulnerability to the crayfish plague pathogen (Gruber et al. 2014; Martín-Torrijos et al. 2017). Plague resistant populations may be a long time chance for conservation of indigenous European crayfish and their conservation considerably depends on effective captive reproduction and juvenile rearing methods.

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ABBREVIATIONS

AFDM	ash-free dry mass
AI	artificial incubation
ANOVA	Analysis of Variances
°C	degree Celsius,
C	Carbon
CF1	crayfish feed 1, Edelkreps (Nofima A/S, Tromsø, Norway)
CF2	crayfish feed 2, Kråkebolle (Nofima A/S, Tromsø, Norway)
Chiro	larvae of chironomidae, Diptera
Chl a	chlorophyll a
CL	standard carapace length measured from the tip of the rostrum to the end of the carapace
CP	cold period
$\delta^{13}C$	ratio of ^{13}C to ^{12}C stable isotopes in the sample in relation to the standard reference material Vienna Pee Dee Belemnite
$\Delta^{13}C$	discrimination factor for ^{13}C
$\delta^{15}N$	ratio of ^{15}N to ^{14}N stable isotope in the sample relative to the concentration in the reference material atmospheric nitrogen
$\Delta^{15}N$	discrimination factor for ^{15}N
Det	detritus
DM	dry mass
DNA	deoxyribonucleic acid
EST	early spring trial
F	degrees of freedom
FLT	Fluorescent Light Tube
LST	late spring trial
LED	Light Emitting Diode
ln	logarithmus naturalis
m^2	square meter
MI	macroinvertebrate insects
n	number of replicates
N	Nitrogen
NH_4^+	Ammonium in its ionised form

NO_2^-	Nitrite in its ionised form
NO_3^{2-}	Nitrate in its ionised form
NSL	natural sunlight
p	p-value in null hypothesis significance testing
PE	polyethylen
Peri	periphyton
pH	potential of Hydrogen
RNA	ribonucleic acid
SGR	specific growth rate
SD	standard deviation
stage I	juvenile crayfish after hatching before the first moult
stage II	juvenile crayfish after the first moult, first independent stage in astacid crayfish
t	time period in days
W_f	final weight
W_i	initial weight
WW	wet weight

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LED = light emitting diodes; FLT = fluorescent light tube; NSL = natural sunlight; SGR = specific growth rate; tot. = total experimental duration.

Appendix

Table 1: Results of the stable isotope analysis from crayfish muscle tissue, commercial feeds and naturally occurring food items (Chapter III). CF1 = high protein crayfish feed, CF2 = low protein crayfish feed, Det = detritus, dark = dark control; LED = Light Emitting Diodes; FLT = fluorescent light tube; NSL = natural sunlight.

Table 2: Isotopic signatures for carbon (δ13C) and nitrogen (δ15N) and the respective discrimination factor calculated following Caut et al. (2009) of the naturally occurring food items in chapter III. Data represent means ± SD (n = 3).

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APPENDIX

Table 1: Results of the stable isotope analysis from crayfish muscle tissue, commercial feeds and naturally occurring food items (Chapter III). CF1 = high protein crayfish feed, CF2 = low protein crayfish feed, Det = detritus, dark = dark control; LED = Light Emitting Diodes; FLT = fluorescent light tube; NSL = natural sunlight.

Sample	Sample ID	δ13C	C (ug)	δ 15N	N (ug)	sample (mg)
dark	LL2-P	-21,12816	467,99335	10,59194	134,84235	1,045
dark	LL4-P	-21,18175	453,96495	10,45752	134,20716	1,073
dark	LR2-P	-21,15281	454,79064	10,24437	136,11225	1,056
LED	LL3-P	-21,12693	486,94475	10,42032	144,35123	1,116
LED	LL5-P	-21,18124	453,13920	10,60249	135,47738	1,045
LED	LR3-P	-21,23615	503,39789	10,23727	146,88095	1,132
NSL	LL7-P	-21,02023	447,35723	9,42745	131,66484	1,035
NSL	LL8-P	-20,78673	451,48751	9,21339	134,84235	1,04
NSL	LR7-P	-20,90248	458,91817	9,13046	138,01591	1,047
FLT	LL1-P1	-21,04221	517,36378	10,17957	153,19421	1,201
FLT	LR1-P	-21,08238	454,79064	10,10232	136,74696	1,072
FLT	LR4-P	-21,26775	463,86919	10,30351	136,74696	1,041
dark	LL4-1	-20,98896	453,13920	10,47672	139,28424	1,079
dark	LL4-2	-21,12130	434,95717	10,19578	127,20972	1,025
dark	LL4-3	-21,17590	444,05189	10,71212	127,20972	1,024
dark	LL4-4	-21,12658	446,53098	10,19093	134,20716	1,031
dark	LL4-5	-21,15866	450,66158	10,24967	137,38152	1,076
dark	LL4-6	-21,12202	437,43828	10,43264	128,48340	1,018
dark	LL4-7	-21,28891	428,33817	10,65982	121,47036	1,03
dark	LL4-8	-21,17239	480,35664	10,39139	137,38152	1142
dark	LL4-9	-20,90443	510,79380	10,62402	150,67079	1,146
dark	LL4-10	-20,94146	461,39396	10,22123	141,18554	1,066
NSL	LL8-1	-20,58483	475,41298	9,50941	141,81899	1,093
NSL	LL8-2	-20,83384	448,18341	9,21834	134,84235	1,041
NSL	LL8-3	-20,70593	519,00566	9,27073	157,60412	1,172
NSL	LL8-4	-20,79683	455,61627	9,08114	132,30066	1,037
NSL	LL8-5	-20,81425	514,07928	8,99938	153,19421	1,16
NSL	LL8-6	-21,12654	471,29157	9,23593	139,28424	1,05
NSL	LL8-7	-20,62790	461,39396	9,23918	138,65015	1,05
NSL	LL8-8	-20,71159	489,41429	9,20367	140,55193	1,056
NSL	LL8-9	-21,01104	462,21909	9,08187	136,11225	1,026
NSL	LL8-10	-21,03655	473,76460	9,57080	135,47738	1,035
Baetidae	Cd1	-23,36439	673,86669	3,93567	117,63703	1,257
Baetidae	Cd25	-21,68874	646,31723	4,40559	126,57264	1,242

Sample	Sample ID	$\delta^{13}C$	C (ug)	$\delta^{15}N$	N (ug)	sample (mg)
Baetidae	Cd35	-21,35453	621,13686	5,12087	114,43826	1,162
Chironomidae	Chiro	-20,98026	361,09989	2,50172	68,59066	0,771
Chironomidae	Chiro	-20,98026	361,09989	2,50172	68,59066	0,771
Chironomidae	Chiro	-20,98026	361,09989	2,50172	68,59066	0,771
Caenidae	Cr3-1	-19,67688	522,28869	5,03153	116,99760	1,135
Caenidae	Cr3-2	-19,88247	16,99125	1,70021	3,41665	0,05
CF 1	E1-1	-23,04512	704,57343	10,28177	138,65015	1,507
CF 1	E1-2	-23,16668	706,18712	10,33919	136,74696	1,52
CF 1	E1-3	-22,99382	423,37134	10,26806	84,82477	0,961
CF 2	Kb1	-24,43374	611,37381	5,93313	54,61605	1,581
CF 2	Kb2	-25,26635	603,23120	5,04847	48,06112	1,493
CF 2	Kb3	-24,65344	395,18433	5,88998	38,85776	0,983
Periphyton	Peri1	-17,85513	468,81800	3,25722	30,35018	1,953
Periphyton	Peri2	-17,51605	715,86413	3,41041	46,68259	2,985
Periphyton	Peri3	-17,90082	1041,19404	2,93173	67,93925	4,03
Detritus	InD1	-28,47040	1180,76786	0,52643	42,01668	2,968
Detritus	InD2	-28,42738	1668,25425	0,62390	55,27067	4,096
Detritus	InD3	-28,69761	1955,37715	0,39352	78,99183	4,971

Table 2: Isotopic signatures for carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) and the respective discrimination factor calculated following Caut et al. (2009) of the naturally occurring food items in chapter III. Data represent means ± SD (n = 3).

	$\delta^{15}N$	$\delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$
CF 1	10.296 ± 0.038	-23,069 ± 0.089	0.863 ± 0.012	0.691 ± 0.010
CF 2	5.624 ± 0.499	-24,785 ± 0.432	2.316 ± 0.155	0.885 ± 0.049
Peri	3.000 ± 0.244	-17,757 ± 0.210	3.070 ± 0.076	0.091 ± 0.024
Det	0.515 ± 0.016	-28,532 ± 0.142	3.905 ± 0.036	1.308 ± 0.016
MI	4,007 ± 1.332	-20,93 ± 1.230	2.819 ± 0.414	0.449 ± 0.139

Table 3: Detailed range of days and degree days required embryonic development of noble crayfish under different temperature regimes in chapter I during artificial incubation (AI), since the water temperature dropped below 4 °C and since the last mating was observed. CP = cold period.

	10 days CP		24 days CP		38 days CP	
	days	degree days	days	degree days	days	degree days
AI						
until hatching	63-73	973-1149	73-83	1017-1192	87-93	1091-1192
until moulting	71-79	1114-1255	82-89	1174-1295	95-101	1226-1327
water < 4°C						
until hatching	73-83	1003-1179	83-93	1047-1222	97-103	1121-1222
until moulting	81-89	1144-1285	92-99	1204-1325	105-111	1256-1357
last mating						
until hatching	93-103	1133-1309	103-113	1177-1352	117-123	1251-1352
until moulting	101-109	1274-1415	112-119	1334-1455	125-131	1386-1487

Table 4: Summary of studies that assessed performance of juvenile noble crayfish using a range of rearing systems, feeds, lighting conditions and densities.

	Experimental conditions						Crayfish performance			
	rearing system	lighting	food	temperature [°C]	period [days]	density [Ind/m²]	survival [%]	initial weight [g]	final weight [g]	SGR [%/day]
Keller 1988	plastic basins outdoor	natural conditions Augsburg, Germany	naturally occurring food items, commercial feeds, carrots, fish	15 - 26	111	800	49	n.s.	0.632	n.s.
						600	65		0.702	
						400	74		0.785	
						200	86		1.054	
Pursiainen et al. 1983	plastic basins outdoor	natural conditions Evo, Finland	zooplankton, fish, vegetables, shrimp waste	up to 26	81	300	50.3	0.038	0.084	0.98
						100	58.5	0.038	0.100	1.20
	ponds		naturally occurring food items	up to 22	81	300	32.4	0.038	0.166	1.82
						100	67.5	0.038	0.221	2.17
Tamevičiene 1988	Net-cages in a lake	natural conditions Lake Baka, Russia	zooplankton, *Chara*, *Elodea,* fish	17 - 23	95	n.s.	n.s.	0.022	0.227	2.49
Policar et al. 2004	AQ MI	12:12 L:D source n.s.	live zooplankton	20.5 ± 0.2	90	400	47.2	n.s.	0.305	n.s.
	AQ AI						26. Feb		0.207	
Kozák et al. 2007	AQ	n.s.	frozen zooplankton	20.6 ± 1.2	56	333 / 164	n.s.	0.044	0.230	2.94
Kouba et al. 2010	AQ	12:12 L:D source n.s.	frozen chironomids, chladophora	14.31 ± 0.62	90	single	63.9	0.020	0.089	1.63
				20.49 ± 0.73			44.4	0.020	0.189	2.47
Chapter I	AQ 10 dCP	12:12 L:D source FLT	commercial feeds, frozen chironomids	20.1 ± 0.3	100	125	85	0.036	281.1	1.88
	AQ 24 dCP						90	0.038	293.8	1.91
	AQ 38 dCP						82.5	0.038	331.7	2.03
Chapter II	AQ	12:12 LD FLT	frozen zooplankton	19.0 ± 1.1	28	400	n.s.	0.045	0.085	2.22
Chapter III	plastic basins indoor	dark	naturally occurring food items, commercial feeds	12.7 - 17. 8 (16.4 ± 2.0)	53	190	77.4	0.035	0.083	1.66
		LED					77.2	0.035	0,083	1.65
		FLT					78.7	0.035	0.088	1.76
		SUN					75.6	0.035	0,122	2.37
	plastic basins indoor	dark	natural occurring food items, commercial feeds	18.2 - 23.7 (21.1 ± 1.2)	58	160	80.8	0.035	0.325	3.62
		LED					84.8	0.035	0.349	3.74
		FLT					89.9	0.035	0.374	3.86
		SUN					90.4	0.035	0.554	4.54

AUTHOR CONTRIBUTIONS

CHAPTER I

Embryonic development, eclosion and performance of juvenile noble crayfish *Astacus astacus* (L. 1758) in response to shortened cold period

LEHMANN K, TORNO J, JESKE H, BRENDELBERGER H

I developed the concept and the experimental design for the study. I set up and performed the experiment with assistance of Helmut Jeske and Johann Torno. I analysed the data and wrote the manuscript. All authors commented on the first draft of the manuscript that is published in "*Invertebrate Reproduction and Development*".

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CHAPTER II

RNA/DNA ratio is an early responding, accurate performance parameter in growth experiments of noble crayfish *Astacus astacus* (L.)

GRIMM C, LEHMANN K, CLEMMESEN C, BRENDELBERGER H

I developed the concept for the study. I developed the experimental design with assistance of C. Clemmesen. C. Grimm performed the study under my supervision and analysed the samples under supervision of C. Clemmesen. I analysed the data and wrote the manuscript together with C. Grimm. C. Clemmesen and H. Brendelberger assisted in preparing the manuscript. C. Grimm and myself equally contributed to the study that is published in *Aquaculture Research.*

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CHAPTER III

Nursery of noble crayfish *Astacus astacus* (L. 1758): Effects of stocking time and lighting on performance of early-hatched juveniles

LEHMANN K, JESKE H, BRENDELBERGER H

I developed the concept and the experimental design for the study. I set up and performed the experiments with assistance of Helmut Jeske. I analysed the samples and the data. I wrote the manuscript that is prepared for submission to *Journal of Applied Aquaculture*.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel:

Noble Crayfish Aquaculture – Embryonic Development and Juvenile Performance

mit Ausnahme der Beratung durch meinen Betreuer selbstständig erarbeitet und verfasst habe. Die Abhandlung ist nach Inhalt und Form meine eigene Arbeit.

Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.

Diese Arbeit wurde weder ganz noch zum Teil an einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Bereits veröffentlichte Manuskripte wurden kenntlich gemacht. Dies ist mein bisher einziges Promotionsverfahren und mir wurde kein akademischer Grad entzogen.

Westensee, den 20.01.2020

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Kai Sören Lehmann