Restoration of Unio crassus rivers in the Luxemburgish Ardennes LIFE11 NAT/LU/857

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Rearing protocol for *Unio crassus*

LE GOUVERNEMENT DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère du Développement durable et des Infrastructures
Département de l'environnement

LE GOUVERNEMENT DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Agriculture, de la Viticulture et du Développement rural

CHAMBRE D'AGRICULTURE LUXEMBOURG

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CONTENT

1 INTRODUCTION

Freshwater mussels are important indicator species in our rivers and lakes. Their presence indicates an intact habitat with a good water quality (Brim Box et al., 2006) and their filter capacity provides an important ecological function to our freshwater ecosystems.

Although the ecological importance of many freshwater mussels is still unknown, the class Bivalvia contains the most endangered species of all freshwater organisms (Neves et al., 1997). Most of the freshwater mussels in Europe are classified as endangered (Bauer & Wächtler, 2001). Within this group is the thick shelled river mussel, whose decline was well documented. The thick shelled river mussel is listed as endangered in Luxembourg and most European countries (Nagel, 1988), it is also specified in annex II of the directive (92/43/EEC). Furthermore it was listed in the IUCN (World red list, www.redlis.org). The thick shelled river mussel was common throughout its distribution range until the mid of the 20. century (Ehrmann, 1933; Jäckel, 1962), often in very high population densities in rivers of different types (Schnitter, 1922; Tudorancea and Gruia, 1968). It was present in tributaries of nearly all rivers (Nagel, 1988) and was described as most common Unio spiecies (Geyer, 1927; Hochwald & Bauer, 1990; Zwanziger, 1920). Therefore Zettler & Jueg (2007) concluded, that approximately 90% of the German thick shelled river mussel population was lost during the last decades. Also in Luxembourg there was a dramatically decline in the populations (Groh & Weitmann, 2004). Except for small remaining populations in the rivers Our and Sauer, the thick shelled river mussel became extinct in Luxembourg.

1.1 The thick shelled river mussel

The thick shelled river mussel (*Unio crassus*, Philipsson, 1788) (figure 1) is a mussel species that lives primarily in freshwater but sometimes also in brackish water. This mussel species was common in large rivers like for example Elbe, Rhein and Donau. In the meantime its appearance in Europe is restricted to smaller rivers and head waters.

Figure 1: Adult thick shelled river mussel on the ground of the river Our

1.2 Anatomie

The thick shelled river mussel has a bulbous, egg-shaped shell, which can be colored from yellow-green to green-brown to dirty brown (Figure 1). It can reach a length of up to 10 cm. The shell can be 2.5 cm

wide und 5 cm high. Inside the shell, the mussel possesses two strong muscles for closing the shells. Two pairs of gills in the inside are responsible for respiration and function also as filtration apparatus that supplies the mussel with food. Furthermore the female animals exhibit brood chambers (marsupia) in the gills, in which the larvae maturate. The muscular foot of the thick shelled river mussel is mostly of white to yellow shade, sometimes orange. The foot serves as anchorage and alignment of the mussel, additionally the mussel can move with it on the river ground.

1.3 Reproductive cycle

The thick shelled river mussel need females and males and becomes fertile at the age of 4 to 5 years. In spring, the males release their sperm into the water which are taken up by the females to fertilize the eggs. The larvae mature in the brood chambers of the gills within the next 4 to 6 weeks (dependent from the water temperature). Healthy and fit females can produce between 50.000 and 100.000 larvae, which are called glochidia. Between May and June the glochidia are released over the siphon of the female mussel. In the water the glochidia of around 0.2 mm are just able to survive a few days without host, therefore they must find a suitable fish host as soon as possible.

When a host fish inhales larvae-containing water, the glochidia attach immediately to the gills of the fish. The larvae are surrounded by a cyst that is formed by the hosts tissue within two days and mature to juvenile mussels within 10 to 35 days (dependent on the water temperature). The juvenile mussels exhibit a foot, digestive system, nervous system and rudiment gills. The mussels drop of the gills, fall on the river ground and live subsequently in the interstitial pore system until they develop into fertile mussels within the next few years (Gum et al., 2012; Hochwald, 1997). The reproduction cycle is illustrated in figure 2.

For the thick shelled river mussel, different host fishes could be identified, like bullhead (*Cottus gobio*) (Hochwald, 1997), brown trout (*Salmo trutta*)(Engel, 1990), chub (*Leuciscus cephalus*), minnow (*Phoxinus phoxinus*), rudd (*Scardinius erythrophthalmus*) (Bednarczuk, 1986; Engel, 1990; Hochwald, 1997; Maaß, 1987), stickleback (*Gasterosteus aculeatus* und *Pungitius pungitius*) (Engel, 1990; Hochwald, 1997) and pope (*Gymnocephalus cernua*) (Maaß, 1987).

In the region of the Eifel and the Ardennes, minnow, chub and bullhead are the most important host fish. As host fish acquire immunity after the first infection with larvae (Hochwald, 1997), mainly young fish are required for the preservation of the thick shelled river mussels.

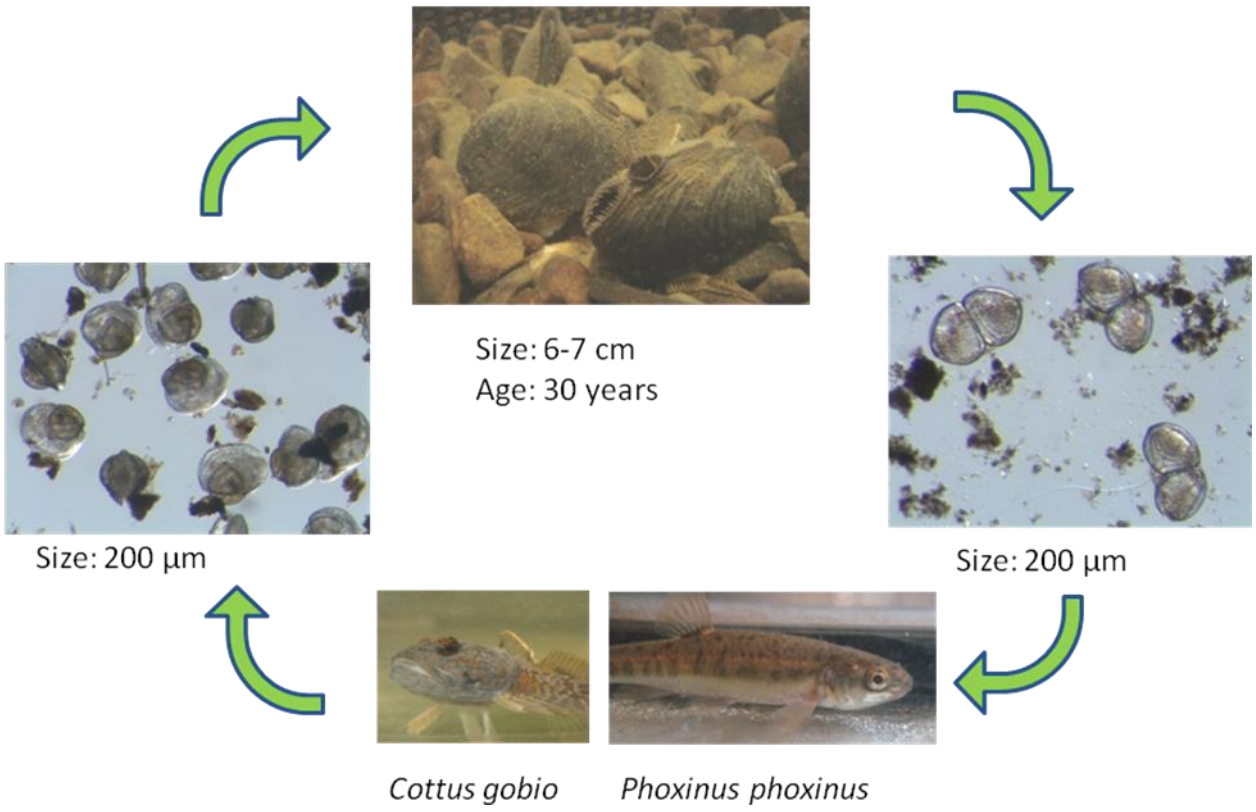


Figure 2: Reproductive cycle of the thick shelled river mussel (*Unio crassus*)

1.4 Nutrition

Young mussels can move around with their foot in the interstitial pore system. Furthermore the ciliated foot transports food particles in direction to their mouth (pedal feeding). When the filter apparatus is fully matured, the gills start to overtake this function. The food of the thick shelled river mussel includes living or dead particles, for example bacteria, protozoa, algae and fine dead plant material (detritus). Adult animals can filter 3 to 4 liters of water per hour. Rivers with large healthy mussel populations are supported in their self-cleaning capacity due to the filtration activity of the mussels.

1.5 Natural habitat

The thick shelled river mussel colonizes small ditches as well as larger rivers. It prefers habitats with sand or gravel in which the adult animals live nearly completely buried (just the two siphons remain visible). The substratum can be coated by a layer of mud or sludge, but especially the interstitial pore system should not be clogged for the juvenile mussels. The thick shelled river mussel often located close to the banks of rivers and streams. These should not be too flat or steep. A natural shore vegetation with native deciduous trees creates additional microhabitats with its rootage, these are used as habitat by the mussels and young host fish.

The thick shelled river mussel is very sensitive and needs clean and nutrient-poor water. Otherwise the requirements in temperature, pH, hardness and water velocity are less high than for instance for the freshwater pearl mussel (*Margaritifera margaritifera*).

1.6 Conservation of the thick shelled river mussel

There are four generel strategies for mussel conservation. These include a) the creation of protected areas, b) the transfer of adult mussel from rivers with healthy populations into rivers with endangered populations, c) the release of host fish infected with glochidia and d) the rearing of juvenile mussels (Ziuganov *et al.*, 1994). In areas with a strong decline of populations, rearing is the only feasible option to protect the mussels (Gum *et al.*, 2011; Preston *et al.*, 2007; Ziuganov *et al.*, 1994) and the likelihood to reach sexual maturity is higher, for example for freshwater pearl mussels, when they live under controlled conditions in captivity during their critical life span as juvenile mussels (Bolland *et al.*, 2010). Furthermore the rearing can be the last possibility („last-minute rescue-tool“) to preserve the genetically potential of mussels (Gum *et al.*, 2011).

2 METHODS

2.1 Production of juvenile mussels for rearing

2.1.1 Infestation of host fish with glochidia of the thick shelled river mussel

The minnow that were used for infestation with glochidia were caught by electrical fishing in spring (normally in April) in the river Our. The minnow was used, as it was tested before in different infestation experiments along with other fishes (brown trout (*Salmo trutta*), minnow (*Phoxinus phoxinus*), bullhead (*Cottus gobio*)) from the river Our and it showed the best results concerning the number of juveniles that could be obtained. Using brown trout's and bullheads, the infestation was less successful and fewer juvenile mussels excysted (Data not listed).
Minnows were set in a tank of 200 liter that was aerated. They were fed with fish pellets (Biomar Inicio Plus, 0.8 mm, Plaidt, Germany) and had the chance to acclimatize in the new setting. To prevent stress, the minnows were not measured, but their total length was estimated to be between 5-10 cm.

Fifty adult thick shelled river mussels were kept in captivity during spawning time. The mussels were placed in gravel containing baskets (layer of gravel approximately 3-4 cm) in a flow through channel that was supplied with river water (Our). Mussels were not fed additionally.
All mussels used were tested concerning their gravidity. This can be done by careful opening of the valves. Swollen gills (Marsupia) indicate gravidity. Every day during spawning time, the gravel was searched for glochidia within white or orange slimy packets that look a bit like corncobs if unripe. The packets were taken with plastic pipettes and put in a beaker with river water. After visual inspection with a stereo microscope, the infestation of host fish was only conducted if most glochidia were free (without egg shell) and agile (clapping of the shells). Normally the first glochidia release occurs between the end of April and mid of May for the river Our. Before the glochidia were given to the water bucket containing the minnows, their number was determined by five times counting all larvae in 100 µl water sample. On the basis of this and the volume of the beaker, the total number of larvae was calculated.

For the infestation, the minnows were caught with nets and set in an aerated bucket of 15 liter. The number of fish was dependent of the number of glochidia (200 glochidia per fish). The glochidia were given to the bucket with fish and over a period of 45 minutes the water was moved carefully by hand every 5 minutes. Directly after infestation, the minnows were set back into the 200 liter tank, where they stayed during the collecting period of the juvenile mussels.

2.1.2 Collecting of juvenile mussels for rearing in captivity

Juvenile mussels were collected in the net of a „juvenile mussel collection station“ (see figure 3) after dropping-of the fish gills. The number of host fish was between 100 and 200 fish. The mesh size of the collecting net (Retch, Düsseldorf, Germany) was approximately 68 µm due to the small size of the mussels (approximately 200 µm). The first juvenile mussels from the river Our could normally be collected between end of May and mid of June.

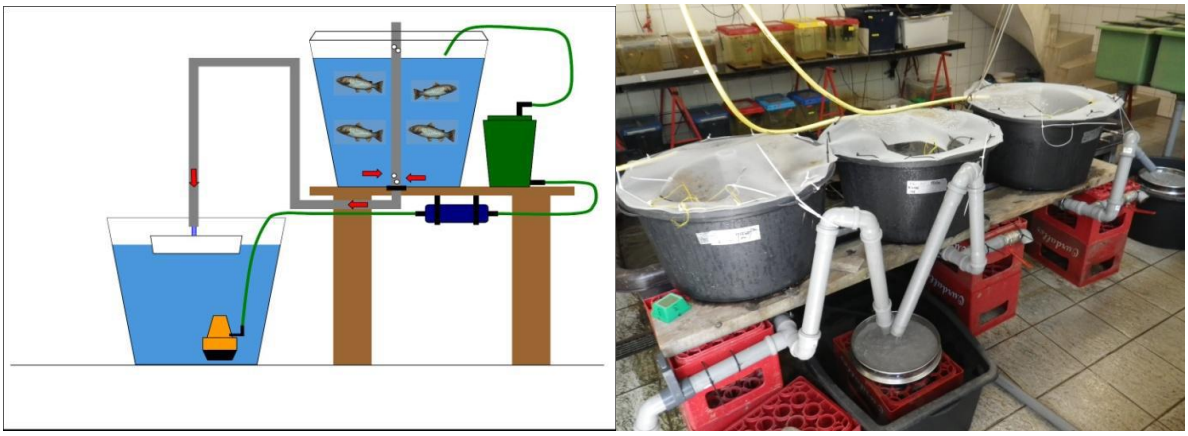


Figure 3: Juvenile mussel collection station: Left: schematic view: Glochidia get from the fish tank over a tube in a collection net (mesh size 68 µm). Right: Fish tank with minnows that function as „juvenile mussel collection station".

2.1.3 Excystment period

In one experiment from the year 2012 with 110 infested minnows, one excystment period was observed in detail. For this purpose all freshly dropped juvenile mussels were collected every 2-3 days from the collection nets, counted, photographed and their length was measured with the computer software ImageJ.

2.2 Food for rearing the thick shelled river mussel

2.2.1. Detritus

The detritus that was used for rearing the juvenile mussels was collected in a wet meadow close to Wilwerdange, Luxembourg. Compared to detritus from the river Our it is more easy to collect in higher quantities. Detritus particles were dispersed in the meadow by stepping on a wet spot with the feet. The detritus was collected with a beaker when the concentration was as high as possible (see figure 4).

Fresh detritus was collected every two to three weeks and stored in an aerated bucket in the basement of the rearing facility at the Mill of Kalborn, Luxembourg. Directly before the detritus was used as food for the mussels, it was sieved through a net with a mesh size of 180 µm. The detritus is a natural mixture of different components, dependent on for example the season, temperature, rainfall and plants in the collection area. Under normal circumstances more than 50% of the algae in the detritus are diatoms, followed by green algae and fragments of algae. Furthermore it contains organic material like zooplankton, different bacteria, fungi and sediment. An analysis of the detritus in the frame of the project „Schutz und Erhalt der Flussperlmuschel in NRW", Germany, showed for example a dry weight of 1.1%, 48.9% ash (from dry weight), a pH of 6.5 (measured in the water), a conductivity of 90 µS cm^{-1}, a TOC of 225 000 mg kg^{-1} and a total nitrogen content of 11 400 mg kg^{-1} (measurements from July 2010).

Figure 4: Detritus. Left: Freshly collected detritus. Right: Wet meadow in which detritus was collected by stepping in wet places with the feet.

2.2.2 Algae

Algae that were used for feeding juvenile mussels were ordered from the company (Reed Mariculture Inc., Campbell, California, USA). One product used is called Nanno3600 (Nanno) and contains *Nannochloropsis* sp. with a diameter of 1-2 µm. The other product is Shellfishdiet1800 (SFD) that consists of a mixture of different algae: *Isochrysis* sp., *Pavlova* sp., *Thalossiosira weissflogii* and *Tetraselmis* sp.. The cells have a diameter of 4-20 µm. Nanno was direcly frozen in small portions after reception and unfrozen as required. The mixture SFD was stored in the fridge (7°C) and approximately weekly, 50 ml were removed for regular use and stored in a centrifugation tube (Roth, Karlsruhe, Germany). After the expiration date, SFD was not used anymore.

2.2.3 Nutrient composition of detritus and algae

The nutrient composition of detritus, SFD and Nanno, that were used to feed juvenile thick shelled river mussels, were determined in the public research center Gabriel Lippmann (now LIST), Luxemburg. For this, the total protein content, the total lipid content, the total carbohydrate content and the ash content in % from dry weight were analyzed. Furthermore there was already data from the manufacturer of the algae (Reed Mariculture Inc., Campbell, California, USA). Due to the complicated analysis methods, the amino acid composition was not determined. As the lipid content in detritus is very low, the proportion of saturated and unsaturated fats was also not investigated.

2.2.3.1 Proteins

The protein content of the samples was analyzed with the method of Kjehldahl. Initially the total nitrogen content was determined. For this method, two exactly weighted samples were solubilized by boiling in an open Erlenmeyer flask with an overage of sulfuric acid for double measurement. Herein the organic parts of the sample were removed and the nitrogen was changed into ammonia sulfate. Thereafter, the nitrogen in the solution was determined with the titrimetric method with 0.1 molar hydrochloric acid. As the nitrogen content is connected with the protein content of biological samples, it could be calculated with the empiric factor of 6.25.

2.2.3.2 Lipids

The analysis of the total lipid content was conducted by Soxleth-Extraktion, a method for determination the lipid content of food and animal food. As for this purpose a big sample quantity was needed, just a single measurement could be done. The exactly weighted sample was lyophilized (Christs, Alpha 2-4 LSC, SciQuip Ltd., Newtown, UK). After this the dried sample was extracted with a degreasant (petroleum ether, Merck, Germany) in a Soxleth-Extractor (Extractor Unit E-816, Büchi Labortechnik AG, Flawil, Swiss). The lipids were solved from the detritus within the solvent vapor. Finally the lipids were freed from the solvent by distillation. The quantity was determined by gravimetric method.

2.2.3.3 Carbohydrates

The quantity of carbohydrates was measured with the method of Edwards. For conducting a triple measurement, three exactly measured samples were lyophilized (Christs, Alpha 2-4 LSC, SciQuip Ltd., Newtown, UK) and afterwards 0.5 ml 80 % ethanol was added and mixed in a vortex mixer. The sample was heated up to 60°C and centrifuged with 12 000 g in a micro centrifuge. The supernatant was removed. This extraction was repeated twice. The collected supernatant was dried in a vacuum centrifuge (Speedvac Plus, Savant Instruments, Farmingdale, USA) to prepare it for the analyze of the soluble carbohydates. After drying the samples, they were resuspended in 1 ml of deionised water. One aliquot of 50 ml was put in a tube for the micro centrifuge, each. These were cooled on ice and anthrone reagent (0.2% anthrone in 70% concentrated sulphuric acid) was added. The mixture was vortexed and cooled on ice for ten minutes and after that heated up to 85 °C. The samples could be analyzed by measuring their absorption at the wavelength of 600 nm (Labtech FLUOstar Optima microplate reader, BMG Labtech, Mornington, Autralia). A glucose-standard-curve in anthrone was prepared that was used to determine the sample values. Triple measurements were made of each sample with a regression coefficient of at least 0.99.

2.2.3.4 Ash

The ash content of the sample was determined by weighing three samples exactly and conducting a lyophilization (Christs, Alpha 2-4 LSC, SciQuip Ltd., Newtown, UK). Thereafter the samples were ashed several times in a muffle furnace (Barnstead 62700 Furncace, Alpha multiservice, Conroe, USA) at 550°C under addition of 2 ml hydrogen peroxide (30%) (Roth, Karlsruhe, Germany). Finally the ash content was analyzed by the gravimetric method after cooling down. The quantity of ash was calculated from the weight difference of the sample before and after ashing.

2.3 Rearing of the thick shelled river mussel

2.3.1 Rearing in plastic boxes

Juvenile thick shelled river mussels were collected within a two week long excystment period. They were rinsed from the collection sieves every 1-3 days on eight days altogether. The number of the collected mussels was counted under a stereo microscope and after taking pictures, their length was measured with the software ImageJ. To prevent food concurrence, only 100 mussels (chosen randomly) were placed in one plastic box. If there were less than 100 mussels in the collection nets, they were put together in one plastic box. In common, there were eight plastic boxes, one for each collection day.

The water volume and the food concentration were adapted to the size respectively the age of the mussels. Table 1 shows the water volume and the feeding composition and concentration, which were used for different ages of the mussels. Mussels were fed during the weekly water exchange.

The lids of the boxes were loosely closed but not closed tightly to allow air circulation. The boxes contained river water (Our, Luxemburg) and were stored at 17-18 °C in a climatic chamber (Grand cru, Liebherr, Germany).

Table 1: Food mixture and volume of the boxes for different age classes of thick shelled river mussels (with a maximum of 100 individual per box)

Age of mussels	Volume of river water in the boxes	Quantity of Shellfishdiet1800 per 1 ml water	Quantity of Nano3600 per one ml water	Quantity detritus per box
0 - 30 days	500 ml	ca. 24 000 cells	ca. 1 836 000 cells	25 ml
30 -110 days	500 ml	ca. 48 000 cells	ca. 3 772 000 cells	25 ml
110 - 120 days	500 ml	ca. 72 000 cells	ca. 4 608 000 cells	25 ml
120 - 200 days	1600 ml	ca. 72 000 cells	ca. 4 608 000 cells	25 ml
200 days - 1 year	4000 ml	ca. 72 000 cells	ca. 4 608 000 cells	25 ml

The number and the length of the surviving individuals was determined after 110 days and after one year. The length of the mussels were furthermore regularly measured at different dates. A linear slope (x-axis: age in days, y-axis: length in mm) was constructed from all length measurements with Microsoft Excel. The regression coefficient (R^2) of the linear slope was calculated by linear regression. Each linear slope of growth included six to seven measurements within 110 days and 9-10 measurements within a year. Furthermore we calculated, using the regression line, after which time period the mussels reached a length of one millimeter.

2.3.2 Rearing in aquaria

An aquarium (Figure 5) consisted of a plastic box (Sunware, Q-line box, 40x30x26 cm) with a layer of sand (Rosi's Aquarienkies, white, 0.1 - 0.9 mm, Quarzverpackungswerk Rosnerski, Königslutter, Germany) with a height of approximately 0.5 cm, in which the mussels could settle. A pump (Swordfish 200 Multifilter, Flamingo, Geel, Belgium) created a current of water in the aquaria.

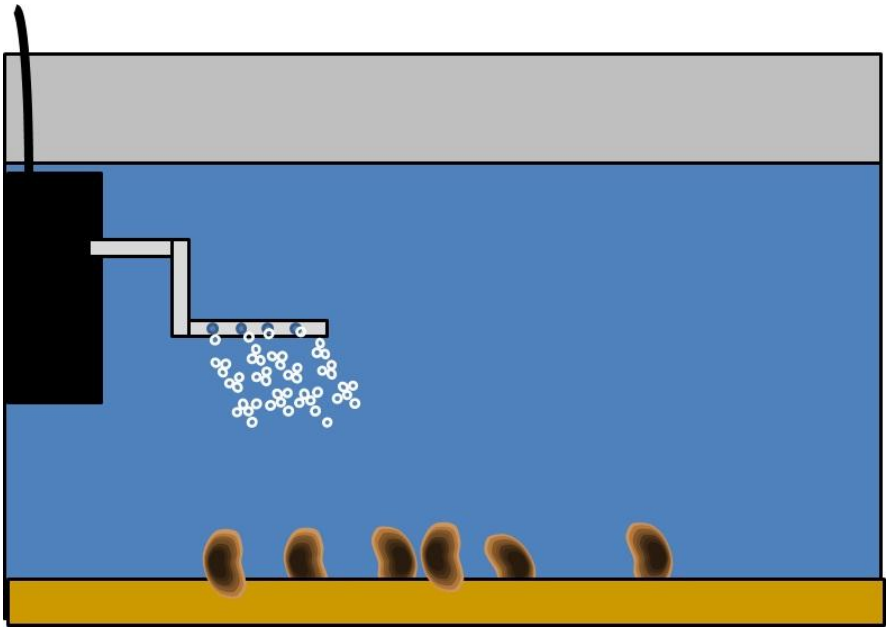


Figure 5: Schematic drawing of an aquaria with sand and pump.

60 thick shelled river mussels with a maximum age of 24 hours after excystment with an average length of 0.22 ± 0.02 mm were placed at the end of June 2013 into six aquaria each. Feeding occurred five times weekly on the working days with algae. At the beginning, the juvenile mussels received 200 µl SFD and 2 drops of Nanno per aquaria. Three month later the amount of SFD was increased to 280 µl and after two additional months to 350 µl per work day. The amount of Nanno stayed over time the same (2 drops). When the tubes of the pump were clogged with dirt, it was cleaned mechanically under tap water. This was necessary all 3-4 weeks. The sand in the aquaria was cleaned twice during the experiment (January and April) by removing all mussels from the aquaria and by rinsing the sand with tap water three times. After cleaning the aquaria were filled with Our water again and the mussels were set back.

2.3.3 Rearing in a flow-through channel with gravel baskets

Different mussel sizes and quantities (compare table 2) were placed in baskets (FIAB Teichpflanzenkorb 230x230 mm, Conrad, Essen, Germany) that were filled with gravel. The gravel was collected in the river Our and was sieved to the size of 2-20 mm. The layer of gravel had a height of approximately 5 cm. The gravel baskets were set in the flow-through channel that was streamed by river water continuously. When too much sediment topped the gravel and single stones were not visible anymore, the baskets were cleaned by gentle shaking them, to remove the covering of the mussels. A reduction of sediment in the river water was also achieved by using a drum filter that sorted particles bigger than 100 µm out. A flow-through channel was illustrated in figure 6. The mussels were not fed additionally and received the natural food from the river with the water. Furthermore the mussels lived under natural conditions concerning for example the temperature or pH with its natural fluctuations throughout the days or year.

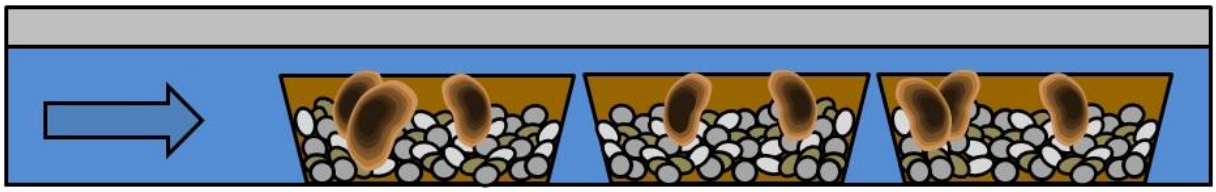


Figure 6: Flow-through channel with gravel baskets

In total, 77 mussels were reared in four baskets in one flow-through channel. Different mussel sizes and quantities (compare table 2) were set in different baskets. The counting and the measurement of the mussels happened at different dates. The mussels were controlled (removed) as infrequently as possible as it was possible to squeeze them with the gravel during removal.

Table 2: Different length and numbers of thick shelled river mussels, that were reared over months in gravel baskets in a flow-through channel.

Basket	A	B	C	D
Beginning of the experiment	July 11	July12	September 12	September 12
Duration in month	23	10	20	9
Length at the beginning of the experiment (in mm)	5.99	15.13	11.1	10.2
Standard deviation	1.01	2.21	1.7	2.2
Number	17	10	30	20

2.3.4 Rearing in a channel with gravel boxes

Similar to the rearing in a flow-through channel, the mussels were placed in gravel containing boxes with lids (Sunware, Q-line Box). From the boxes side-pieces of approximately 10 x 10 cm were cut out and covered by gauze (mesh size of 2 mm) to allow water to flow through the box (compare figure 7).

Figure 7: Gravel box for rearing mussels in a rearing channel

The gravel was sampled in the river Our and had the size of approximately 2-30 mm. The height of the gravel layer was around 5 cm. The gravel boxes were put in the rearing channel at the Mill of Kalborn for a semi-naturel release into the wild, which has a width of 50-60 cm and runs over a meadow. The water comes from the mill stream. Approximately 3-5 m upstream the side where the gravel boxes were placed, a small artificial pond, in which different water plants like waterweed (*Elodea nuttallii*) and bulrush (*Typha latifolia*) grow, was located. In common, 32 thick shelled river mussels (compare table 3) were set in gravel boxes in the rearing channel behind the pond at the Mill of Kalborn, Luxembourg. The mussels were not fed additionally and received the natural food from the river Our/the mill raceway and the pond. Furthermore the mussels lived under natural conditions concerning for example the temperature or pH with its natural fluctuations throughout the days or year.

Table 3: Number and length of the thick shelled river mussels that were reared in gravel boxes in the rearing channel.

Basket	A	B	C
Length at the beginning of the experiment in cm	2.32	1.98	1.85
Standard deviation	0.23	0.32	0.21
Number	12	10	10

3 RESULTS

3.1 Collecting of juvenile mussels for rearing in captivity

3.1.1 Excystment period

The analyzed excystment period from the year 2012 had a duration of 14 days. During this time period 833 juveniles could be collected in common from 110 minnows. The number of excysted mussels per collection day was presented in figure 8. Most mussels excysted in the first half of the excystment period.

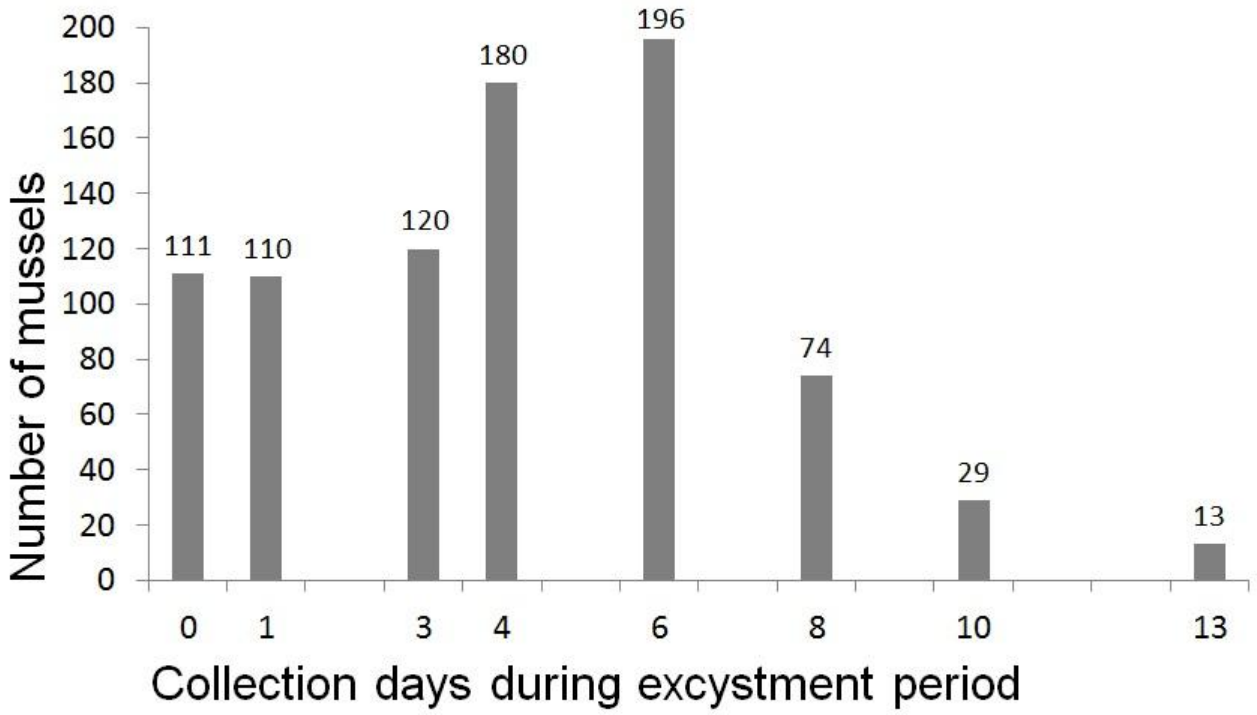


Figure 8: Number of collected juvenile thick shelled river mussels during one excystment period

3.2 Food for rearing the thick shelled river mussel

3.2.1 Nutrient composition of detritus and algae

Additionally to the values that were given from the manufacturer of SFD and Nanno, the nutrient composition for both algae mixtures was complemented with our own analysis. Detritus mostly consisted of inorganic substance (ash) and furthermore contains a much lower content from proteins, lipids and carbohydrates, when compared to both algae mixtures. The content of proteins, lipids, carbohydrate and ash of Nanno, SFD and detritus was illustrated In table 4. The values from the manufacturer and the own analysis differed partly distinctly.

Table 4: Nutrient composition of Nanno, Shellfishdiet und Detritus (in % of the dry weight)

	Nanno*	Nanno**	Shellfishdiet*	Shellfishdiet**	Detritus**
Proteins	58.6	36.53	52.0	15.57	6.22
Lipids	14.5	0.438	16.1	1.173	0.003
Carbohydrates	20.0	3.484	22.0	1.551	Not detectable
Ash	5.9	27.53	9.9	68.88	65.73

*as analyzed by Reed Mariculture Inc.
**own analysis

3.3 Rearing of the thick shelled river mussel

3.3.1 Rearing in plastic boxes

The examined excystment period of the thick shelled river mussel had a duration time of 14 days. The freshly excysted mussels had on different collection days different average length between 0.21 and 0.24 mm (figure 10 a). After 110 days, the same mussels had an average length between 0.60 and 1.72 mm and between 6.7 and 8.8 mm after one year (figure 10 b). Juvenile thick shelled river mussels after a few weeks of growth are illustrated in figure 9.

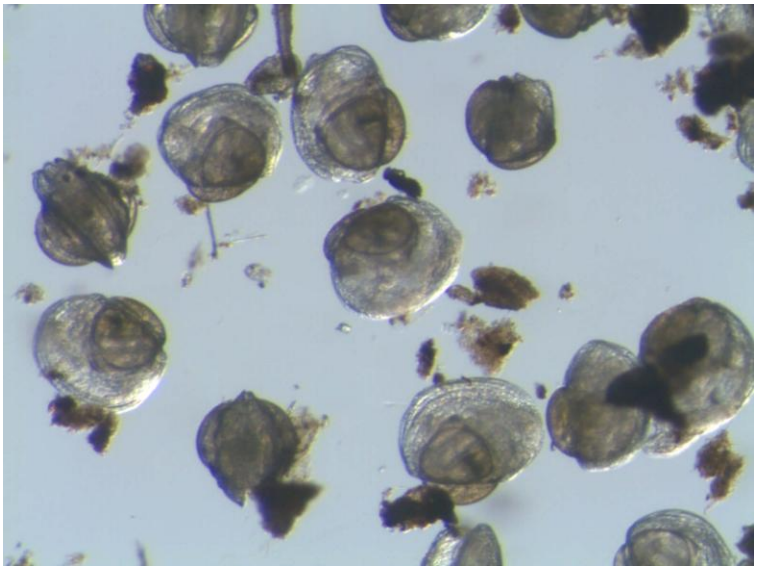


Figure 9: Juvenile thick shelled river mussels after a few weeks of growth

The survival rate after 110 days was, for different collection days, between 27 and 100% and between 0 and 92 % after one year (Figure 10 c). After one year 152 of 833 mussels had survived. The pearson-correlation-coefficient showed a significant correlation between collection day and the length of the mussels at the age of 110 days ($P=0.036$), as well as between the collection day and the survival rate after 110 days ($P<0.021$). The length of the mussels on the collection day was not significantly correlated with the collection day. All mussels that were collected after the first day of the excystment period showed a high linear growth (calculated by linear regression) with a regression coefficient (R^2) between 0.97 and 1.00 during 110 days (figure 10 d). Mussels collected on the first day of the excystment period in contrast, showed in the first 110 days a lower growth with a flattened growth curve ($R^2=0.75$). The mussels that where collected from collection day 3 to the last collection day reached an average size of 1 mm between the age of 58 and 76 days. (This age was calculated by linear regression. R^2 was between 0.98 and 1). The mussels from collection day 1 (R^2 of 0.97) reached an average size of 1 mm after calculated 105 days and the mussels from collection day 0 (R^2 of 0.75) only after 231 days.

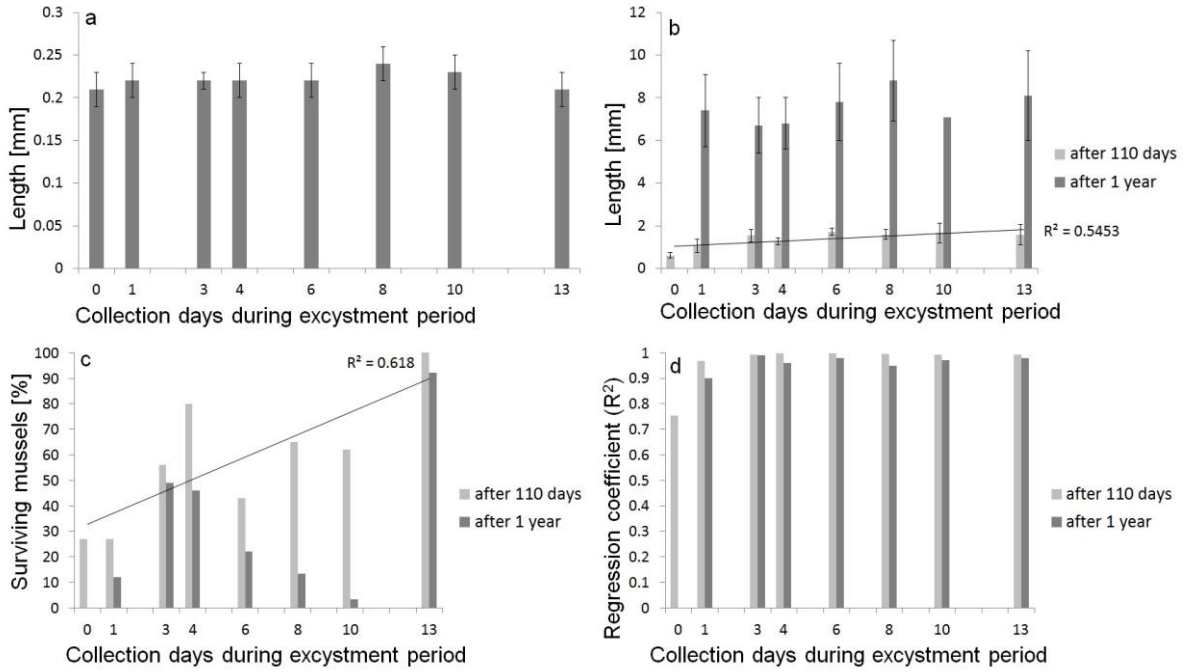


Figure 10: a: Average length (in mm) of juvenile thick shelled river mussels directly after excystment, that were collected during one excystment period. b: Average length (in mm) of the juvenile mussels at the age of110 day and one year. c: Survival rate (in %) of the juvenile mussels at the age of 110 days and one year. d: regression coefficients (R^2) of the linear slopes of the mussels after 110 days and one year.

3.3.2 Rearing in aquaria

The average survival rate in six aquaria, in which 60 juvenile thick shelled river mussels were placed directly after excystment, was 59.17 % within one year. The surviving mussels in all aquaria reached an average size of 1.07 cm (± 0.20cm) during that time period. The average length of the mussels as well as their survival rates were illustrated for each aquarium in figure 11.

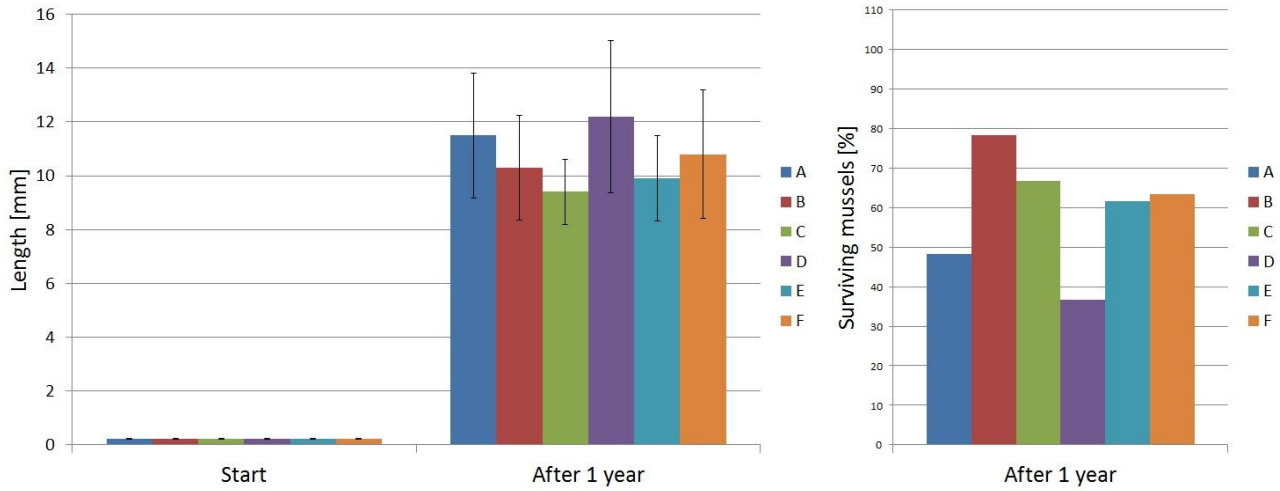


Figure 11: Average length and survival rates of thick shelled river mussels, that were reared in aquaria (A-F) for a time period of one year. (Number per aquaria: 60 mussels)

3.3.3 Rearing in a flow-through channel with gravel baskets

Different numbers and length of thick shelled river mussels were placed in four gravel baskets in a flow-through channel. The growth- and survival rate of the mussels were illustrated in figure 12.

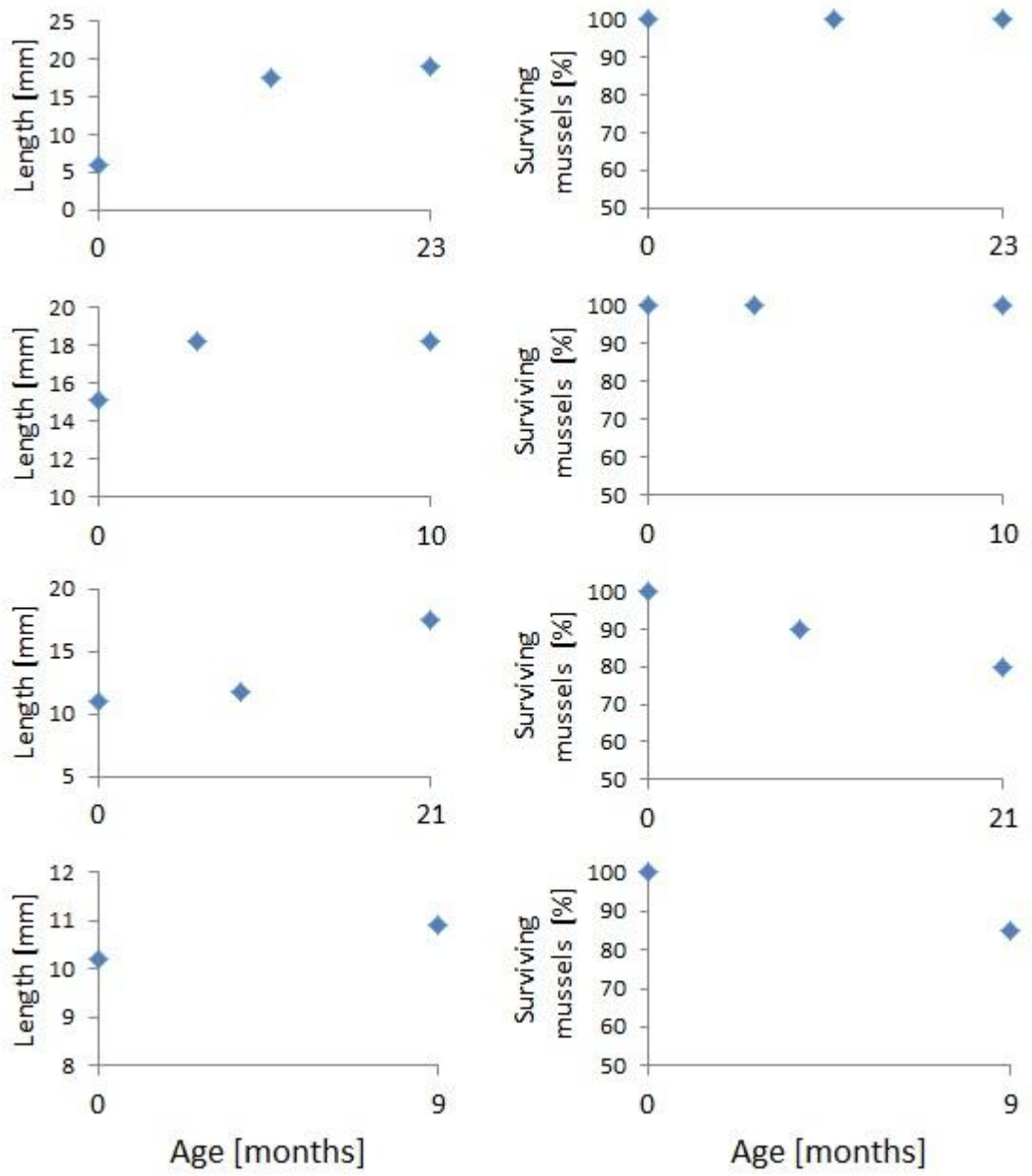


Figure 12: Average length and survival rates of thick shelled river mussels that were reared in four gravel baskets (A-D) in a flow-through channel.

The first of the four gravel baskets (A) was the only basket that was occupied with mussels in the year 2011. It showed an average growth of 11.48 mm in the first year. In the following 11 month, the average growth was lower (1.63 mm). In basket B the mussels showed from the beginning of July a

growth of 3.07 mm (average) within three month. In the following colder month (October to May) no growth was visible. Basket C as well as basket D were filled with mussels in September 2012. During the cold month until the end of May the mussels did not grow much (lesser than one mm in average). The mussels in basket C showed in the following 12 month a growth of 5.88 mm in average. In all four baskets, a survival rate of 85-100% in the first 9-12 month could be achieved. In basket A all mussels had survived after 23 month.

3.3.4 Rearing in a rearing channel with gravel boxes

From all thick shelled river mussels, that were reared in the rearing channel, 80 – 100 % survived per gravel box (average of all gravel boxes: 93.75 %). The surviving mussels in the different gravel boxes had reached different average length of 2.52 (±0.17) to 2.86 cm (±0.2) after one year. This means that the mussels showed a growth between 0.54 and 0.69 cm per year per gravel box (in average 0.63 cm per gravel box per year). Data was illustrated in figure 13.

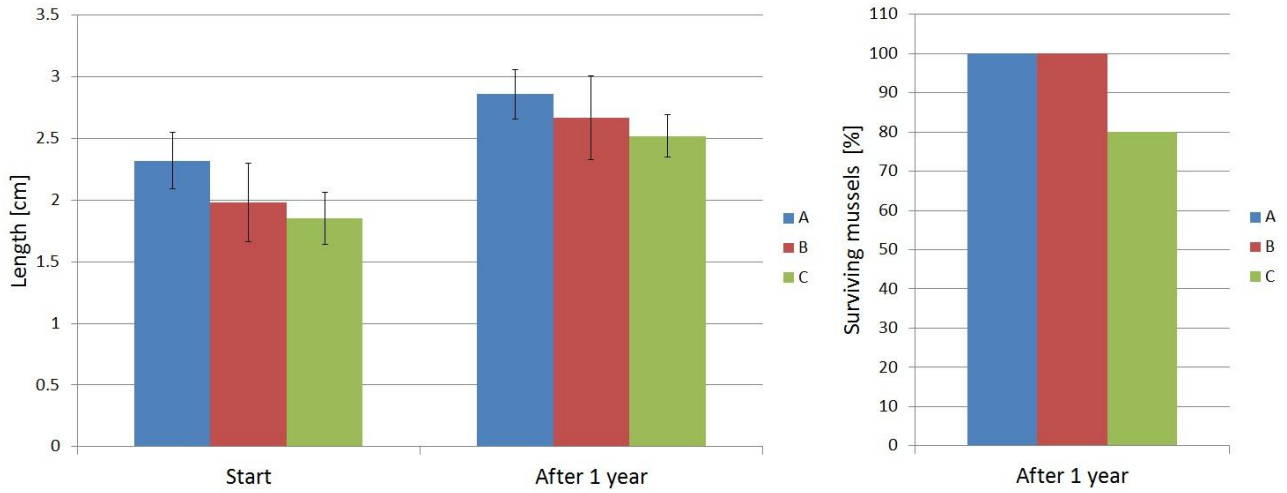


Figure 13: Length and survival rates of juvenile thick shelled river mussels, that were reared in gravel boxes (A-C) for a time period of one year in the rearing channel of the Mill of Kalborn (Luxembourg).

4 DISCUSSION

4.1. Collection of juvenile mussels for rearing in captivity

4.1.1 Infestation of the host fish with glochidia of the thick shelled river mussel

Referred to Hochwald (1997), female thick shelled river mussels are able to release glochidia several times a year. This behavior was also observed for the mussel species *Unio pictorum* and *Unio tumidus* (Fleischauer-Rössing, 1990). The multiple releases of larvae during one summer can make it possible to perform an infestation of host fish at different time points and therefor excystment periods at different time points during one summer, too. In our case this multiple release of larvae during one year could not be observed for the mussels from the rivers Our or Sauer.
For the thick shelled river mussel, different host fishes could be defined like bullhead (*Cottus gobio*) (Hochwald, 1997), brown trout (*Salmo trutta*)(Engel, 1990), chub (*Leuciscus cephalus*), minnow (*Phoxinus phoxinus*), rudd (*Scardinius erythrophthalmus*) (Bednarczuk, 1986; Engel, 1990; Hochwald, 1997; Maaß, 1987), stickleback (*Gasterosteus aculeatus* und *Pungitius pungitius*) (Engel, 1990; Hochwald, 1997) and pope (*Gymnocephalus cernua*) (Maaß, 1987). All these fishes were identified as possible host fish for the thick shelled river mussel. Due to electrical fishing, it is known, that wild minnows, bull heads and brown trout are naturally infested with glochidia. The minnow was chosen as a host fish as it is existent in an adequate number in the Our river an can be caught easily by electrical fishing. Furthermore Taeubert et al. (2011) and Taeubert et al. (2012) identified the minnow (and *S. cephalus*) as the best host fish for the thick shelled river mussel, whereas the bullhead and the brown trout were lesser suited and approximately 90% of the glochidia can be lost within sixteen days on these fish species (Taeubert et al., 2011). Similar results were obtained in experiments at the river Our.

The minnows that were used for the rearing of the thick shelled river mussel at the river Our, had probably already contact with *Unio crassus* glochidia in the river during the years before. For instance the natural prevalence of wild fish was 60% (data not shown) in two consecutive years. During a second infestation the following year there could have been a loss of glochidia due to a immune response of the fish host (Hochwald, 1997). However in our case the infestation was successful and enough mussels could be collected for rearing. There seems to be no problem to take minnows from the home river of the adult mussels for breeding.

4.1.2 Excystment period

During the excystment period, there was a „peak“ in which most mussels could be collected. Collecting mussels during this short time period is very effective, as high quantities of mussels can be obtained for rearing. Most juvenile mussels could be collected in the first half of a two week long excystment period. To obtain enough mussels for rearing, this week should not be missed.

4.2 Food for rearing the thick shelled river mussel

Detritus was taken as food after a personal advise (Lange, pers comm. 2009). Furthermore, this method was already used by other mussel breeders, for example Hruska (1999, 2001). As an supplemental food source, the algae mixtures SFD and Nanno were used, as algae were already successfully used for rearing different juvenile mussels species (Hudson & Isom, 1984). For example algae species like *Nannochloropsis* sp. (species in Nanno) were used in circulating culture systems as primary food source (Gatenby *et al.*, 1996; O'Beirn *et al.*, 1998; Barnhart, 2006). A mixture of different algae species normally results in higher growth- and survival rates compared to feeding one

single algae species (Brown *et al.*, 1997; Romberger & Epifanio, 1981). In this work, manufactured algae for rearing were preferred, as the culture of large quantities algae is time consuming and hence expensive.

4.2.1 Nutrient composition of detritus and algae

The values for the nutrient composition of the manufacturer and the own measurements differed clearly. Unfortunately the manufacturer did not state which methods were used, therefore no direct comparison can be made between the results. For example the usage of another empiric factor during protein determination could have led to different results, although the method after Kjehldahl (Bock, 1972; Hoegger, 1998) was also used. For the carbohydrate analysis it is not known, if the manufacturer included the polysaccharides, these were not included in our own analysis. And for the lipids different methods could have led to different results. The manufacturer furthermore had the possibility to conduct the analysis on freshly raised algae, whereas the own analysis were performed on older products. For example the SFD sample was already stored in the fridge at 7°C for over a month and the Nanno sample was frozen for several month. This could have led to an altered nutrient composition.

The detritus consisted mainly of inorganic substance (ash) and a much lower part of proteins, lipids and carbohydrates compared to the algae mixtures. Therefore it can be concluded, that detritus is less suitable as the only food source. However, as an additional food source, detritus could offer some substances that are not existent in SFD and Nanno. For example other algae species, bacteria and fungi could content additional nutrients. Furthermore the clay minerals in the detritus have the properties to bind different minerals like potassium, calcium and magnesium and to form a depot for these elements (Kelley, 1942). Additionally compounds like amino acids and sugars can bind on the surface of these particles (Weiss, 1969) and juvenile mussels that take in fine sediments, can be supplied with minerals and essential nutrients that are needed for an optimal growth and survival.

4.3 Rearing of the thick shelled river mussel

Whereas the freshwater pearl mussel (*Margaritifera margaritifera*) was already successfully reared by some researchers, there is no or only little experience in rearing the thick shelled river mussel which can be used to compare the results of this work. To our knowledge, the infestation of minnows with glochidia and to rear the collected juvenile mussels in the laboratory was one of the first experiments done in this direction.

4.3.1 Rearing in plastic boxes

With this method, it was already possible to rear freshwater pearl mussels successfully (Eybe et al., 2013). To prevent food competition in the boxes, a maximum of 100 individuals were placed in one box each. The mussels in the boxes reached average length between 0.60 to 1.72 mm after 110 days. Compared to this, juvenile freshwater pearl mussels reared under the same conditions reached an average length of 0.86 to 1.48 mm. This shows that thick shelled river mussels grow faster under the same conditions (number of individuals, food quantity, 500 ml boxes) as they started with an average length of 0.21 und 0.24 mm compared to the freshwater pearl mussels of 0.31 and 0.38 mm. This was also confirmed after one year, in which the thick shelled river mussels reached an average length of 6.7 to 8.8 mm, but the freshwater pearl mussels just a length of approximately 4 mm. When the mussels were collected later during the excystment period, the rearing was more successful. They were significantly longer and had better survival rates after 110 days when compared with mussels that were collected at the beginning of the excystment period. Thus, the survival rates at the first two collection dates were the lowest with 27% each after 110 days. The later survival rates were with up to 100% much better. The value of the regression coefficients of the linear slopes (growth over time) that were calculated for different collection days, correlated not just with the growth of the mussels, but also with their survival rate. Thus, the lowest regression coefficient of just 0.75 was calculated for the mussels on collection day 0 and after one year no mussels from this collection day were still alive. For all other collection days, the regression coefficient was between 0.97 and 1. It seems as if a low regression coefficient is linked to a lower fitness of the mussels.
There was no significant increase in mussel length during the different collection days. The reason is most likely that glochidia of thick shelled river mussels generally grow rarely or not at all on their host fish (Maaß, 1987; Taeubert, 2011). Thus, the juvenile mussels during the excystment are not often longer than the glochidia during the infestation (glochidia approximately 200 µm, juvenile mussels 0.21 to 0.24 µm).
The age of the mussels when they reached an average length of 1 mm (calculated by use of the linear regression) was between 58 and 76 days (without the first two collection days). If the size of 1 mm is as important for the survival of the thick shelled river mussels as for the freshwater pearl mussel (Buddensiek, 1991; Lange & Selheim, 2011) is not known. However the handling of larger mussels is much easier, as the mussels become visible with the bare eye.

After one year, fewer than 25% of the collected mussels from the excystment period survived. One reason for this bad survival rate could be a too low food concentration in the boxes. Furthermore as the mussels had no substrate in the plastic boxes they could not sit up and were always on the side. They had to open their valves against gravity to take in food (to filter). Maybe, thick shelled river mussels are dependent on the presence of substrate at this age and on a current of water that brings food particles to the mussels (change from pedal feeding to filter feeding).

Not feeding the host fishes during the excystment period showed no negative effect on the juvenile mussels. Even mussels that dropped-off late during the excystment period, when the fish were already

starving, showed good survival rates after 110 days and a fast growth. Therefor we can recommend to stop feeding the minnows during excystment period as this makes the collection of the were small juveniles from the cleaner collecting sieves much easier.

4.3.2 Rearing in aquaria

Nearly 60 % of all mussels reared in aquaria survived the first year. Maybe the survival rate could have been even better if the mussel had the size of approximately 1 mm before being transferred to the aquaria. Smaller mussels (0.2mm) could be easily covered with sand and die during water exchange as it is possibly more difficult for them to move up to the surface again. The mussels reached an average length of 1.07 cm, which is a good result as they can be set in gravel baskets without problems with this size.

4.3.3 Rearing in a flow-through cannel with gravel baskets

With this method, good survival rates between 85 to 100 % could be achieved within 9-12 month. In two baskets, the mussels grew 5.88 and 11.48 mm which is also a good result. Why the mussels in basket A showed a much higher growth in the first year compared to the last 11 month cannot be explained. Presumably there were fluctuations due to the different weather in both years that led for example to different food concentrations in the river water.
The results furthermore show that the main growth period is in the warm months (approximately June to September), in the cold months (approximately October to May) no or just little growth occurred. The mussels in the baskets C and D had good survival rates although they were placed there in September. Hence, the late transfer seems not to harm the mussels (in contrast to the freshwater pearl mussel). As long as the water temperature does not differ more than 3°C it seems that they can be transferred without problems from boxes with a stable temperature (16-17°C) into a flow-through channel in which the temperature fluctuates by the season.

4.3.4 Rearing in a rearing channel with gravel boxes

In contrast to the freshwater pearl mussel that moves up to several centimeters deep in the gravel layer (own observation), the thick shelled river mussels were always near to the gravel-water surface and visible. Normally their siphons were directly at the surface of the sediments that regularly covered the gravel as mud. With this characteristic, the thick shelled river mussels have certainly an advantage compared to the freshwater pearl mussels, as they are not easily covered by sediments that increase after a heavy rain event in the river water. The danger to be cut off from food or to suffocate in the mud is reduced. During the first year 93.75 % of the mussels survived in the rearing channel. This was a very good result. Also the growth rate (average of 0.63 cm per gravel box) was very satisfactory. The mussels reached after one year an average length of 2.52 (±0.17) to 2.86 cm (±0.2).

5 SUMMARY

The following findings were obtained for the rearing of the thick shelled river mussel:

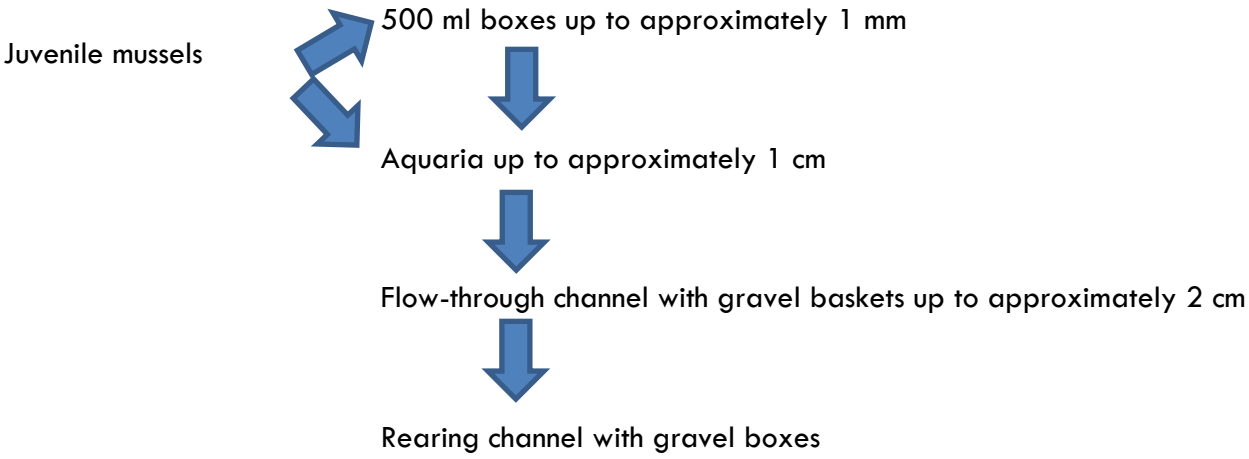
-autochthonous minnows that were caught by electric fishing can be used for the infestation with glochidia.

-The minnows should not be fed during the excystment period, as otherwise the collection nets will be clogged. Mussels seem not to have a disadvantage doing this.

-Mussels that fall during the first few days of an excystment period have a bad survival and grow slowly. Not too much time should be invested for rearing these mussels. Later mussels are fit and suited for intensive care as they grow well and have a high survival chance.

-The short time period in which most mussel fall during the excystment period should not be missed.

- For the rearing of the thick shelled river mussel, the following optimal sequence of holding methods could be established:



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