Application of a five-stage field key for the larval development of the freshwater pearl mussel *(Margaritifera margaritifera* Linné, 1758) under different temperature conditions –

A tool for the approximation of the optimum time for host fish infection in captive breeding

Christian Scheder
Clemens Gumpinger
Daniela Csar
Consultants in Aquatic Ecology and Engineering
Gärtnerstraße 9
A-4600 Wels
scheder@blattfisch.at
gumpinger@blattfisch.at
csar@blattfisch.at

Abstract

As the freshwater pearl mussel (*Margaritifera margaritifera* Linné, 1758) is highly endangered throughout its distribution area, conservation programmes are carried out in many countries, dealing mostly with semi-natural captive mussel breeding. The crucial stage of such breeding projects is the fixing of the ideal time for collecting mature mussel larvae for the infection of the provided host fish. The present study introduces a five-stage field key for the determination of clearly discernible developmental stages of mussel larvae. By means of this key, the developmental progress at any certain instant of time can be determined, and the remaining time till the release of mature larvae can be estimated.

As larval development in poikilothermic animals always depends on the temperature of the surrounding medium, three case studies at markedly different thermic situations are provided for Central European conditions. An average, a comparably cool and a relatively warm summer have been compared with regard to larval development.

In the extraordinarily warm summer of 2007, a succession of two complete reproduction cycles of the normally strictly univoltine freshwater pearl mussel was detected. Reasons and possible effects are discussed.

Résumé

La moule perlière (*Margaritifera margaritifera* Linné, 1758) est au bord de l'extinction bien que protégée. Il y a beaucoup de projets dans tout l'Europe qui essayent à sauver les populations restantes en infectant des truites farios avec des larves du mollusque, qui se développent à l'abri des branchies de ces poissons. Le problème essentiel, c'est trouver le moment exact pour l'infection. Celle recherche-ci présente une clé d'identification pour les stades de développement avec laquelle cinq stades peuvent être déterminés directement sur le terrain. Le progrès du développement peut être identifié à chaque instant quelconque par le biais de la clé pour estimer le temps restant jusqu'à la libération des larves mûres.

Comme le développement des animaux poïkilothermes relève de la température de l'eau, trois études modèles sont mises à disposition. Elles traitent trois situations thermiques qui se distinguent essentiellement l'un de l'autre et qui sont typiques d'Europe centrale. Il s'agit d'une année moyenne, une année relativement chaleureuse et une année comparativement froid.

En été 2007 les températures extraordinairement hautes ont conduit à deux cycles de reproduction particuliers, quoique normalement, les moules perlières soient univoltins. Probables raisons et effets possibles sont discutés.

Zusammenfassung

Da die Flussperlmuschel (*Margaritifera margaritifera* Linné, 1758) in ihrem gesamten Verbreitungsgebiet als höchst gefährdet zu betrachten ist, werden in vielen Ländern Artenschutzprogramme durchgeführt, die vor allem auf die halbnatürliche Nachzucht von Jungmuscheln im Labor ausgerichtet sind. Die sensibelste Phase in diesen Nachzuchtprojekten ist die Festlegung des richtigen Zeitpunktes für die Infektion von Wirtsfischen mit den parasitären Larven der Flussperlmuschel.

In der vorliegenden Arbeit wird ein fünfstufiger Bestimmungsschlüssel für klar gegeneinander abgrenzbare Entwicklungsstadien von Flussperlmuschelglochidien vorgestellt. Mit Hilfe dieses im Freiland anwendbaren Schlüssels kann zu jedem beliebigen Zeitpunkt der Entwicklungsfortschritt der Muschellarven festgestellt und die Zeit bis zum Erreichen der Infektionsreife abgeschätzt werden.

Da die Geschwindigkeit der Larvalentwicklung bei allen wechselwarmen Tieren stark von der Umgebungstemperatur abhängt, werden drei für zentraleuropäische Verhältnisse typische Temperaturszenarien miteinander verglichen. Ein durchschnittlicher, ein vergleichsweise kalter und ein auffällig warmer Sommer wurden in Hinblick auf die Larvalentwicklung in einer natürlichen Flussperlmuschelpopulation miteinander verglichen.

Im außergewöhnlich warmen Sommer 2007 wurden zwei separate, aufeinanderfolgende Fortpflanzungszyklen bei der sonst streng univoltinen Flussperlmuschel beobachtet. Mögliche Gründe für dieses Phänomen und daraus resultierende Effekte werden diskutiert.

Introduction

The freshwater pearl mussel (*Margaritifera margaritifera* Linné, 1758) is one of the most threatened species in the Northern hemisphere (Young et al. 2001), especially in Europe. Once having occurred in lime-poor running waters all over Northern, Central and Western Europe in vast numbers, its populations have declined for several decades and still keep decreasing rapidly. Disillusioning reports of dropping mussel numbers arrive from virtually every country of its distribution area, including amongst others England and Wales (Chesney & Oliver 1998), Northern Ireland (Beasley & Roberts 1996), Scotland (Cosgrove et al. 2000), Ireland (Moorkens 1999), Germany (Vandré et al. 2000), the Czech Republic (Hruška 1998), Spain (Bouza et al. 2007), Latvia (Rudzīte 2005), Belgium (Terren et al. 2006), Luxemburg (Jungbluth 1988) and Austria (Scheder & Gumpinger 2007).

The reasons for the grave situation are manifold. Firstly, the freshwater pearl mussel shows a very complicated and peculiar reproduction mode. The female mussel produces several million of parasitic larvae, so-called glochidia, which are borne within special formations of the parental gills (Young & Williams 1984). After having completed their development inside their mothers´ shells, the larvae are expelled into the surrounding water and are immediately inhaled by brown trout (*Salmo trutta* Linné, 1758), the preferred host fish species for Central European freshwater pearl mussel populations (Wächtler et al. 2001). Once attached to the host´s gills, they cling to the respiratory tissue that soon starts overgrowing the parasite. The glochidia overwinter inside the cysts, undergo metamorphosis in late spring, then disengage from their hosts and drop to the riverbed. For at least five years, they live inside the hyporheic interstitial (Bischoff et al. 1986), before they join their adult conspecifics on the surface of the riverbed. It is obvious that such a meticulous reproduction cycle is highly vulnerable. The crucial stage in the life cycle is the period during which the juvenile mussels live in the interstitial (Geist 1999). Geist & Auerswald (2007) identified the characteristics of the stream substratum, the depth profile of the redox potential, the penetration resistance of the stream bottom and the physical connectivity of free-flowing water and the interstitial zone as substantial factors for successful pearl mussel recruitment. Due to the intensive agricultural use of the catchment areas (combined with overfertilization and the clear-cut of alluvial forests), enormous loads of fine sediments are transported into the river systems, causing heavy siltation effects in the interstitial (Altmüller & Dettmer 1996). The pores in which the juvenile mussels dwell are clogged, and the supply of nutrients and oxygen is cut off. The young mussels hence either suffocate or starve to death, and the populations concerned show a remarkable excess of age.

In all the countries mentioned above, protection projects are currently being carried out in order to prevent the mussel from becoming extinct. As the present study bases on surveys carried out in Austria, the specific situation of the Austrian freshwater pearl mussel populations is depicted below.

In Austria, the distribution area of the freshwater pearl mussel has always been restricted to the northern parts of Upper and Lower Austria, where it used to occur in enormous densities (Gumpinger et al. 2002). Nowadays, only some isolated scattered beds are left, the largest ones not exceeding a few hundred mussels (Scheder & Gumpinger 2008). Most populations lack juveniles, as the natural reproduction has not resulted in a sufficient amount of viable juvenile mussels throughout the past decades – mostly due to the severe siltation of the river beds. In order to conserve the few remaining populations, a large-scale protection programme has been carried out in the Upper Austrian River Waldaist for over ten years, dealing mainly with the support of the natural reproduction by semi-natural breeding. This very population was chosen, as Moog et al. (1993) have described the River Waldaist as "the best remaining freshwater pearl mussel stream in Austria with a population of highest relevance and worthiness of protection". Geist & Kuehn (2005) examined the population´s genetic aspects and proved a closer relationship to the population in the River Kamp in Lower Austria than to any Upper Austrian population. They therefore suggest regarding the populations of the River Waldaist and the River Kamp as a separate conservation unit within the Danube drainage.

In the course of the species protection project, a method for approximating the optimum time for the artificial infection of the host fish has been developed by describing five developmental stages that are easily discriminable in the field.

Material and Methods

Investigations were carried out in the largest remaining Upper Austrian mussel population, which is located in the River Waldaist. Physicochemical characteristics of the river are listed in Tab. 1 (data provided by the Office of the State Government of Upper Austria, Department of Surface Water Management (Linz)).

Between 2005 and 2007, the gestation rate of adult mussels from the River Waldaist and the degree of development of their larvae were monitored every summer. The survey aimed at gaining mature larvae for the semi-natural infection of cultured fish that were to be released into appropriate river systems immediately after infection. In order not to miss the larval release, examinations were carried out at periods constantly decreasing in length. At the commencement of each reproduction cycle, controls were performed once a week. With preceding development, intervals were shortened after each monitoring, until, during the final stage of the observations, daily samples were taken.

Several mussels were taken out of the substrate cautiously, and slightly opened by means of special pliers. If gravidity could be attested – in terms

Tab. 1: Physicochemical characteristics of the River Waldaist (average over 33 months: November 2006 – July 2009).

MQ (m^3s^{-1})	Conductivity ($\mu S\ cm^{-1}$)	pH	Magnesium ($mg\ l^{-1}$)	Calcium ($mg\ l^{-1}$)	Total hardness	DOC ($mg\ l^{-1}$)	Oxygen ($mg\ l^{-1}$)
3,12	102	7,32	1,64	9,33	1,69	6,56	11,30
Orthophosphate ($mg\ l^{-1}$)	Phosphorous Total ($mg\ l^{-1}$)	Potassium ($mg\ l^{-1}$)	Sodium ($mg\ l^{-1}$)	Sulfate ($mg\ l^{-1}$)	Nitrate ($mg\ l^{-1}$)	Nitrite ($mg\ l^{-1}$)	Ammonium ($mg\ l^{-1}$)
0,015	0,035	1,20	6,68	9,21	1,13	0,004	0,44

of the presence of a distinct, yellowish cell mass within the gill tissue – samples of larval material were taken with the aid of a disposable syringe. The samples were then observed employing a hand held optical microscope (Enhelion Micron pro, 160-fold magnification). Micrographs were taken by means of a digital camera (Canon PowerShot G6).

Conspicuous changes in larval morphology, morphodifferentiation and mobility over the course of time were registered. Clearly distinguishable developmental stages were characterized verbally and sketched from micrographs by means of a technical pen.

In order to find out about a correlation between the rate of larval development and the water temperature, a temperature measuring probe (Te.M.P. by blattfisch) was installed in the River Waldaist directly next to the mussel bed.

Results

The development of the glochidia turned out to pass through five stages that are easy to discriminate morphologically in the field by means of basic light microscopy. The exact process of larval development in *M. margaritifera* has been described explicitly by Scharsack (1994) by use of electronic microscopy. But in the course of applied conservation projects, more simply discernible stages that can be distinguished by means of basic equipment can be regarded as a significant work simplification. The five "field stages" are described and pictured below.

Stage 1. The first distinguishable stage can be characterized as a spherical, compact mass of cells without any further differentiation (Fig. 1a). The larva is enclosed within a thin, transparent, globular-shaped egg shell. The first stage is totally immobile. Individuals are often closely attached to their neighbours, forming long strings or even tissues of larvae. At this early stage of development, the larva has already reached its final dimensions of about 40 – 70 μm in diameter. In the course of the following stages, only further differentiation, but no more growth takes place. Stage 1 is the most long-lasting of the five stages described in this study.

Stage 2. As soon as a larva has reached the second stage, distinct constrictions become clearly visible along the median axis of symmetry (Fig. 1b). Thus, the left- and right-hand side of the larva can be distinguished for the first time at this stage. The second stage is still enclosed within the egg shell and fully immobile.

Stage 3. In the third stage, the future mussel shells develop. The typical semi-spherical, hollow structures, in which the body is to be enclosed, are formed. Viewed ventrally or dorsally, the two valves appear drop-shaped (Fig. 1c), as the median and lateral edges of each valve converge and meet at the tip at an acute angle. The final shape of the glochidium is fixed at the end of the third stage. Still, no movement can be detected at this stage, and the larva still lies inside its transparent egg shell.

Stage 4. Hardly any major differentiation occurs between stage 3 and stage 4, but larvae that have reached the fourth stage start moving inside their egg shells. They perform snapping movements by actively opening and closing their shells. As development goes on, the snapping becomes more and more frequent. Membranous structures can be noticed between the valves, being stretched when the larva opens its shells (Fig. 1d). From the dorsal or ventral view, two tooth-like projections can be perceived at the margin of each valve.

Stage 5. Larvae hatch from their egg shells and start moving around freely (Fig. 1e), snapping heavily. The stout spines at the apical end of each shell are now clearly visible. When a strongly diluted solution of sodium chloride is added, the larva closes its shells and does not open them anymore. This reaction is explained by the fact that free fifth stage larvae have to find an appropriate host fish and attach to its gills as quickly and strongly as possible. As in fish a large part of the salt metabolism is performed via the gills (Smith, 1929), a high concentration of ions in the surrounding water indicates the presence of adequate host tissue to the glochidium.

Only stage 5 larvae are capable of infecting host fish successfully. In semi-natural breeding it is therefore inevitable to collect larval material exactly at the right time. If glochidia are gained too early, larvae cannot cling to the host´s gills properly, as they are still encased in their egg shells. Waiting too long for mature larvae on the other hand may result in a total loss of glochidia, as gravid

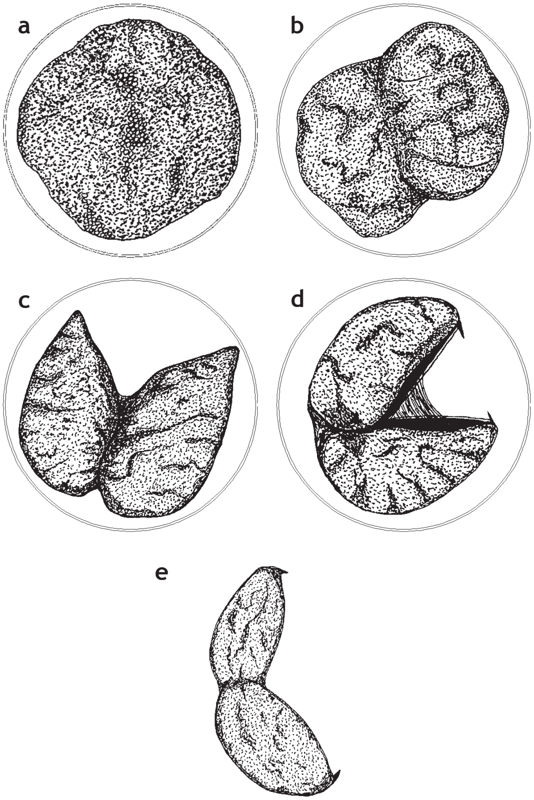

Fig. 1: Developmental stages of the freshwater pearl mussel; a: stage 1 (age: 4 days); b: stage 2 (age: 16 days); c: stage 3 (age: 23 days); d: stage 4 (age: 28 days); e: stage 5 (age: 30 days). Age specification for mean water temperatures of 14.3 °C in August.

mussels of the same population expel their larvae highly synchronically. The observation of the larval development and the assignation to one of the five described stages can help to make a rough estimate of the time that is left until the release of mature, infectious larvae. As the developmental rate in poikilothermic animals always depends on the temperature of the surrounding medium, the prediction of the ideal time for infection must always regard the prevalent temperature regime of the watercourse. The varying time requirement for the completion of development under different temperature conditions is depicted below on the basis of three exemplary thermal situations for Central European conditions.

The years 2005, 2006 and 2007 represented three exceedingly different years regarding the respective water temperature regime (Tab. 2). 2005 can be referred to as an "average" year with air and water temperatures typical of the warm-moderate, Central European climate. The mean water temperature in August amounted to 14.3 °C, the mean annual water temperature to 8.1 °C. In such a year, the first occurrence of stage 1 larvae can be expected at the end of July or beginning of August (Fig. 2, top left). As the first stage is the most long-lasting one in the course of development, stage 1 larvae can be found for about two weeks. Under average temperature conditions, stage 2 glochidia will be present from the middle of August onwards. The rate of development is getting slightly faster with every stage; that is why stage 3 larvae are likely to replace stage 2 larvae within about eight to ten days. The further development proceeds even more quickly. The transition from stage 3 to stage 4 takes place within half a week, so does the final step from stage 4 to stage 5.

The summer of 2006 was, by contrast, remarkably cold. The mean water temperature in August amounted to only 13.0 °C. Though first stage larvae could be detected at the beginning of August (just like in the average summer of 2005), the development of stage 2 larvae was markedly retarded (Fig. 2, top right). They could not be proven until the third week of August, their development thus

Tab. 2: Temperature characteristics of the three different temperature scenarios (2005, 2006, 2007).

	Mean water temperature in August (°C)	Number of days with water temperature:				
		< 14 °C	≥ 14 to < 15 °C	≥ 15 to < 16 °C	≥ 16 to < 17 °C	≥ 17 °C
"Average" temperature scenario (2005)	14.3	11	13	3	3	1
"Low" temperature scenario (2006)	13.0	26	3	1	1	0
"High" temperature scenario (2007)	15.2	6	8	7	10	0

having lasted one and a half time as long as under average conditions. The developmental steps from stages 3 to 4 and stages 4 to 5, respectively, lasted even twice as long as under average conditions. In fact, only very few individuals actually reached the fifth stage, as almost all gravid mussels expelled their larvae prematurely.

The summer of 2007 was, in turn, the summit of an extraordinarily warm period that had already started in September 2006. The water temperature of the River Waldaist never reached the freezing mark in the course of that winter, as it usually does; it actually did not even drop below 2 °C. Especially during the period of egg maturation, water temperatures markedly exceeded average conditions. The mean water temperature in August amounted to 15.2 °C, 2.2 K more than in the cold summer of 2006. Due to the high temperature, first stage larvae already appeared at the end of June, accordingly four weeks earlier than under average conditions. In fact, reproduction started so early that the first stage had already been completed before the survey was started. The stages succeeded relatively fast, the periods between single stages did not exceed one week each (Fig. 2, bottom). As the water temperature remained higher than average, examinations were continued throughout August. At the beginning of August, a second reproduction cycle started within one single season. Due to the convenient conditions, time spans between the stages were markedly lower than at average temperature terms.

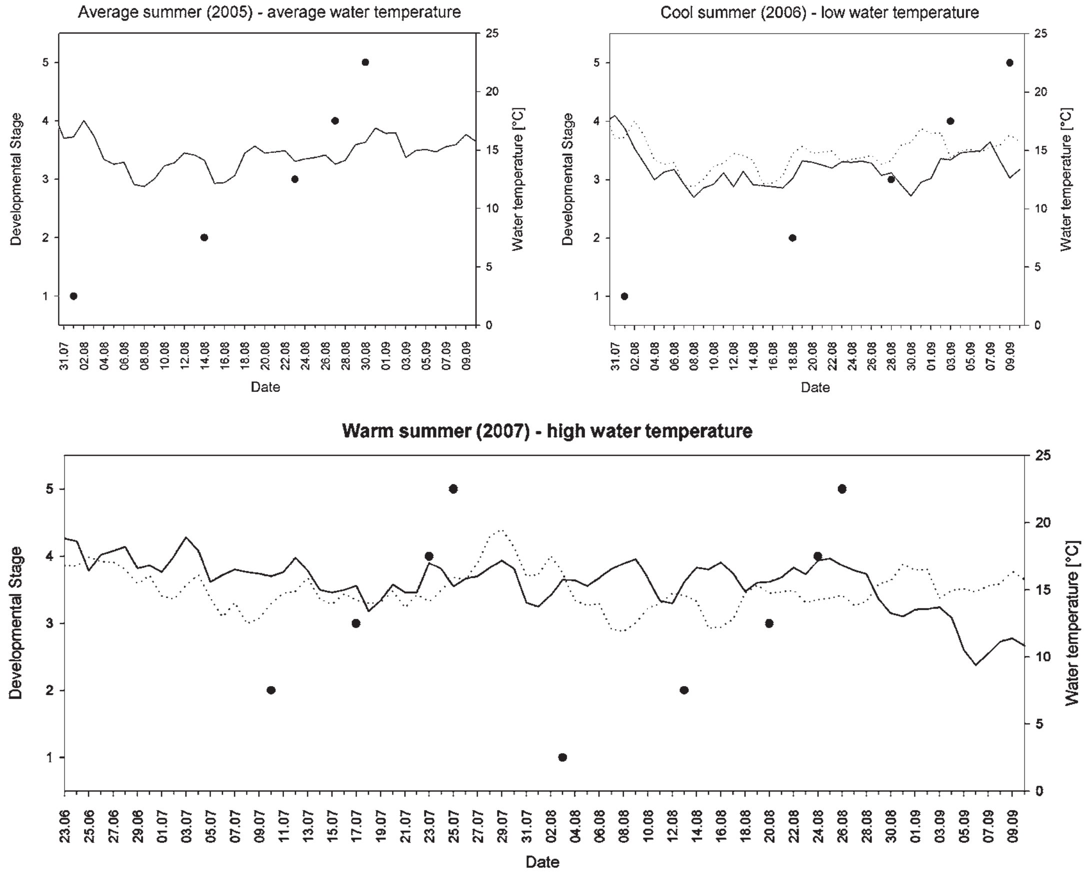

Fig. 2: Developmental course of glochidia of a freshwater pearl mussel population in the River Waldaist at different water temperature regimes (top left: "average" mean temperature, 2005; top right: "low" mean temperature, 2006; bottom: "high" mean temperature, 2007). Dots: First occurrence of respective larval stage. Solid line: water temperature in the respective year. Dotted line: water temperature in the average summer of 2005.

Development rates differed markedly between different temperature conditions. The durations of each larval stage and of the entire developmental course depending on the three temperature situations described above are presented in Tab. 3.

The addition of daily mean water temperatures between the first and the last date of detection of a special stage leads to the degree-days required for the completion of the respective stage (Tab. 3, Fig. 3). The sum of degree-days for the total development ranged from 353 under warm conditions to 530 at low water temperatures. At average terms, the sum of degree-days amounted to 428.

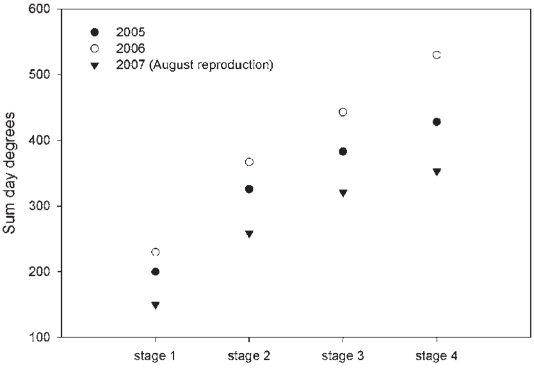

Fig. 3: Degree-days required for the completion of each developmental stage (cumulative graph). Stage 5 is not included, as it complies with the completion of stage 4.

Discussion

Slight fluctuations in developmental patterns are common in natural systems, as the rate of development in poikilothermic species correlates closely to the temperature of the surrounding medium (e. g. Gordon 1984; Pritchard et al. 1996; Lapointe 2001). Minor temperature shifts between single years have always occurred in the warm-temperate climate of Central Europe. Hence, it can be assumed that developmental cycles in freshwater pearl mussel populations have always varied in length, onset and termination throughout the years, as Hastie & Young (2003) confirm exempli gratia for Scottish rivers. Hence, the different developmental rates in average, warm and cold summers presented in the paper at hand are to be considered as natural fluctuations. But the fact that the surveyed population in the River Waldaist performed two complete consecutive reproduction cycles within one single vegetation period in 2007, although the freshwater pearl mussel is regarded strictly univoltine, must be discussed as an absolutely special case. According to the Austrian Central Institute for Meteorology and Geodynamics (ZAMG), the year 2007 can be characterized as one of the warmest years since recordkeeping began. In eleven consecutive months, temperatures above average were recorded. These conditions seem to have influenced the reproductive behaviour of the observed population. It could be detected that coherent parts of the population initiated the generation of glochidia substantially earlier than the rest. As only gravidity was observed and male specimens were therefore not examined for this issue, it is uncertain whether the whole population had split into two separate and independent reproduction groups, or if males generated spermatozoa twice consecutively. Furthermore, the effects of fragmentation into separate reproduction groups with different timing are difficult to estimate. Biological cycles have evolved over long periods of time, always influenced by the prevailing abiotic parameters. In the reproduction cycle of *M. margaritifera* correct timing is a critical success factor. Larvae develop inside their cysts according to the temperature of the surrounding water. As soon as the sum of day degrees reaches a certain value – 1,300 according to Hruška (1998) – metamorphosis takes place and the juvenile mussels drop from

Tab. 3: Durations of larval stages and the entire larval development depending on the prevalent water temperature.

	Duration of development (days)					Degree-days
	stage 1	stage 2	stage 3	stage 4	Total	
"Average" temperature scenario (2005)	14	9	4	3	**30**	**428**
"Low" temperature scenario (2006)	18	10	6	6	**40**	**530**
"High" temperature scenario (2007)	10	7	4	2	**23**	**353**

their hosts. Whether temperature conditions in the river are appropriate for juveniles which derive from the early reproduction cycle and therefore drop earlier, cannot be answered without further investigation. In any case, adverse effects on the reproduction success cannot be ruled out. As the IPCC (2001) states that in the near future the temperature will constantly rise, extreme years like 2007 and further asynchronous reproduction cycles are likely to increase in quantity.

Degree-days are a common means for predicting the duration of a developmental stage under certain – often static – circumstances. They are used in aquaculture in order to calculate the time of hatching for fish eggs that are cultivated at a certain, constant temperature (Bishop 1971; Blaxter 1969). However, the exact determination of degree-days for glochidia of the freshwater pearl mussel in the field must be considered almost infeasible. Firstly, in natural habitats the water temperature fluctuates irregularly, depending, inter alia, on the prevailing weather conditions. Furthermore, the significance of mean daily water temperatures also depends on the length of intervals between measurements and is therefore always afflicted with a certain inaccuracy. Moreover, the exact commencement of gravidity can usually not be detected in conventional conservation projects that mostly target on collecting glochidia and normally start at empirically determined dates. As soon as stage 1 larvae can be found, it is impossible to reconstruct the exact time of their original appearance. In warm summers, few days of inaccuracy can lead to perceivable differences in calculated degree-days. The degree-days given in this paper are therefore only approximate values, but are in accordance with Lange et al. (2008) who state a sum of 450 degree-days for the total development of infectious glochidia.

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