# Study of selected population parameters of weatherfish *Misgurnus fossilis* (Cypriniformes, Cobitidae): early life history and status of ploidy in fish from Lužnice River floodplain area

## Studie vybraných populačních parametrů piskoře pruhovaného, *Misgurnus fossilis* (Cypriniformes, Cobitidae): raná ontogeneze a úroveň ploidie u ryb ze záplavového území Lužnice

## Bořek Drozd

**FACULTY** OF **FISHERIES** AND **PROTECTION** OF **WATERS**
UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

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**Bořek Drozd**

*I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.*

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**Supervisor:**

Assoc. Prof. Dipl.-Ing. Jan Kouřil, Ph.D.
University of South Bohemia in České Budějovice (USB)
Faculty of Fisheries and Protection of Waters (FFPW)
Institute of Aquaculture (IA)
Laboratory of Artificial Propagation of Fish
Husova třída 458/102
370 05 České Budějovice
Czech Republic

**Head of Laboratory of Artificial Propagation of Fish USB FFPW, IA:**

Assoc. Prof. Dipl.-Ing. Jan Kouřil, Ph.D.

**Dean of Faculty of Fisheries and Protection of Waters:**

Prof. Dipl.-Ing. Otomar Linhart, DSc.

**Board of doctorate study defence with referees:**

Prof. Dipl.-Ing. Petr Ráb, DSc. / Assoc. Prof. MSc. Josef Matěna, CSc. – head of the board
Prof. Zdeňka Svobodová, DVM, DSc. – board member
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Assoc. Prof. Dipl.-Ing. Pavel Kozák, Ph.D. – board member
Assoc. Prof. MSc. Jana Pěknicová, CSc. – board member
Assoc. Prof. MSc. Josef Matěna, CSc. – board member
Assoc. Prof. Dipl.-Ing. Tomáš Randák, Ph.D. – board member
Assoc. Prof. MSc. Jan Kubečka, CSc. – board member
Assoc. Prof. MSc. Pavel Stopka, CSc. – board member

Prof. Dr. hab. Ewa Kamler – international referee
Dr. Jörg Bohlen, Ph.D. – referee

**Date, hour and place of Ph.D. defence:**

14th September 2011, at 10 a.m. in a small meeting room of Municipality Vodňany

# CONTENT

# CHAPTER 1

**GENERAL INTRODUCTION**

## 1.1. DISTRIBUTION

Loaches of the genus *Misgurnus* (Osteichthyes, Actinopterygii, Cypriniformes, Cobitidae) exhibit typical Holarctic distribution but only one species of them, the weatherfish *Misgurnus fossilis* (L.) inhabits freshwater habitats throughout Europe (Kottelat and Freyhof, 2007). The *M. fossilis* is spread from northern France (Seine R. basin) to western Russia (Volga R. basin), but absent in Great Britain, Scandinavia and the Mediterranean (Kottelat and Freyhof, 2007).

## 1.2. HABITAT PREFERENCES AND MIGRATIONS

The *M. fossilis* is a benthic hiding fish species inhabiting slowly flowing or stagnant freshwater habitats, especially the secondary arms, isolated backwaters and temporary pools. It prefers localities in river floodplain areas characteristic with high structural complexity, such as habitats overgrown with dense aquatic vegetation and with muddy bottoms where the substratum is mixed with detritus and dead vegetation (Meyer and Hinrichs, 2000; Pekarik et al., 2008). These localities often suffer temperature-, pH- and water level disturbances, as well as high ammonium and sulphate concentrations but low concentrations of oxygen (Meyer and Hinrichs, 2000; Pekarik et al., 2008). The *M. fossilis* inhabits such habitats together with other anoxia-tolerant species, such as mudminnow *Umbra krameri* Walbaum (Kovac, 1997) and crucian carp *Carassius carassius* (L.) (Halacka et al., 1998; Pekarik et al., 2008).

Our unpublished data based on electrofishing (Drozd, Musil and Bláha, 2007–2009) in lowland floodplain pools of Lužnice R. (Elbe R. basin, North Sea drainage, Czech Republic) reported on typical fish composition of localities inhabited by *M. fossilis*. In summary, 18 fishes were recorded with the species importace evaluated up on the frequency of species occurrence in percentage as followed: *M. fossilis* (100%), Northern pike *Esox lucius* L. (72%), *C. carassius*, European perch *Perca fluviatilis* L., tench *Tinca tinca* (L.) and rudd *Scardinius erythrophthalmus* (L.) (57% each). These species were thus considered the dominant or typical species for such localities (i. e. parapotamon and plesiopotamon) within Lužnice R. floodplain area. Spined loach (*Cobitis sp.*), sunbleak *Leucaspius delineatus* (Heckel) and burbot *Lota lota* (L.) at localities with more stable water conditions, i. e. deeper water with higher oxygen concetration, were classified as the additional species occurring in the pools over the entire year (14% for each of them). Season – dependent occurrence was shown for *Leuciscus* species [chub *L. cephalus* (L.), dace *L. leuciscus* (L.), ide *L. idus* (L.), 14% each], i.e. typical riverine fishes spawning there during the mating season in spring. From late autumn to late spring, season – dependent findings of roach *Rutilus rutilus* (L.), bream *Abramis brama* (L.), common carp *Cyprinus carpio* (L.), topmouth gudgeon *Pseudorasbora parva* (Temminck and Schlegel) (14% each) conduced as a result of human activities, especially of pond harvesting: the Třeboň region has been traditional pond fish farming area in South Bohemia since the Middle Ages. Analogous to preceding, finding of further invasive fish, black bullhead *Ameiurus melas* (Rafinesque) (new fish species for open freshwater in Czech Republic; Musil et al., 2008) at one of the localities under study, is probably originating from casual escapees from pond culture as well.

In terms of habitat selection, *M. fossilis* is commonly assumed a hiding species, which uses submerged macrophytes as shelters and burrows into the bottom in winter or on unfavourable water conditions, as summarized by Meyer and Hinrichs (2000). Adults prefer deeper patches in macrophytes, whereas juveniles are usually found close to shore line in very shallow water where they hide among vegetation or detritus. Both the adults and juveniles avoid open water surface areas without any vegetation (Meyer and Hinrichs, 2000).

Compared to related species, *M. fossilis* is nearly a non-migrating species undertaking only short distance migrations (on a scale of tens or hundreds of meters) for over-wintering (Meyer and Hinrichs,

2000). In oriental weatherfish *Misgurnus anguillicaudatus* (Cantor), Saitoh et al. (1988), Naruse and Oishi (1996), Katano et al. (1998) observed regular seasonal changes in migration activity in response to reproduction and feeding opportunities.

## 1.3.   GROWTH AND DIET

To date, we have little information regarding growth, age, as well as food demands and electivity in *M. fossilis*. Weatherfish is a medium aged species, reaching commonly up to 10 (exceptionally 15) years of age, which usually attains the maximum size ca 20–30 cm (rarely 35 cm) and body mass ca 150 g (Susta, 1937; Oliva and Chitravadivelu, 1973; Stranai, 1990). To date, only studies made by Stranai (1990) in individuals originated from the wild and by Bohl (1993) in pond-cultured specimens provide relevant evidence on growth of *M. fossilis*.  The 1-year old juveniles achieve the average total length 85 mm and body mass 3.7 g (Drozd – unpublished data from the nature). Remarkable growth acceleration is described by Bohl (1993) in females during the second year of life, presumably as an effect of later achievement of sexual maturity compared to males. Based on data of Bohl (1993) or our unpublished data originating from the wild populations inhabiting the Lužnice R. floodplain area, fish achieve in that age the average total length and body mass 90–100 mm and 3–5 g or 120 mm and 8.5 g, respectively.

Weatherfish is generally considered a fish with nocturnal activity (searching for prey), which spends daytime hidden in the sediment (Fric, 1904). However, Naruse and Oishi (1994) observed in individuals of related species *M. anguillicaudatus* reared in laboratory conditions the apparently increased activity during daytime as a response to sufficiency of food particles. In *M. fossilis* a similar movement pattern was observed by Drozd (unpublished data). Moreover, *M. anguillicaudatus* shows at the localities of its natural occurrence a highly seasonally and daily dependent activity pattern in relation to sex, individual size and changes of environmental conditions. In summer its activity tends to nocturnality predominantly in males and juveniles, or to diurnality in females. A gradual decline in water temperature during autumn is manifested in diurnality in all individuals regardless of sex and size (Naruse and Oishi, 1996).

Loaches take advantage of well-developed sense of smell for searching suitable food and on the other hand, they do not use eyesight by feeding (Watanabe and Hidaka, 1983). Based on study of Susta (1937), which is the only one relevant literary source showing any data about diet of *M. fossilis* in nature, weatherfish adults feed on benthic organisms, especially on chironomid larvae and molluscs including their early developmental stages as well. In captivity, *M. fossilis* adults are nourished by sludge worms, chironomid larvae and coarse sieved zooplankton (Kouril et al., 1996). A little more information is available about the diet of *M. anguillicaudatus*. Adults feed on chironomids, ephemeropteran and trichopteran larvae. Juveniles smaller than 40 mm prefer chironomid larvae, while those bigger predominantly feed on ephemeropteran larvae (Katano et al., 2003). Ito and Suzuki (1977) suggested to discriminate nutritional contribution of particular diet components one from each other, because of near indigestibility of plant particles (parts of detritus, i.e. diatoms – Bacillariophyceae, green algae – Chlorophyceae) for early developmental stages of *M. anguillicaudatus*. While components of animal origin are fully digestible for them, and thus *M. anguillicaudatus* larvae successively feed on rotifers, chironomids and small crustaceans, in relation to mouth and body size.

To date, however, almost nothing is known about the actual dietary requirements or foraging behaviour of *M. fossilis* during its early life history. Kouril et al. (1996) noted that artificially reared *M. fossilis* larvae and juveniles actively fed on benthic organisms such as chydorids (genus *Chydorus*), cladocerans (genus *Bosmina*) and copepods. Nevertheless, rotifers have not been found in the guts of larvae nor of juveniles, while they were highly abundant in the environment.

## 1.4.  CONSERVATION STATUS

However, *M. fossilis* possesses the unique adaptations, such as outer filamentous larval gills, segmental blood vessels, intestinal breathing (Grieb, 1937; Kryzanovskij, 1949; Kostomarova, 1975), its density decreased rapidly across the entire Europe during the second half of the 20th century  as a response to water quality degradation, along with drying or direct destruction of naturally occurring habitats (Meyer and Hinrichs, 2000).

The *M. fossilis* is nowadays considered either endangered-vulnerable (Lelek, 1987) or least concern species (Kottelat and Freyhof, 2007), it is included in many red lists of endangered fishes in Europe and it is listed under Annex II of Council Directive 92/43/EEC.

## 1.5.  REPRODUCTION

In *M. fossilis* both sexes are easily distinguishable from each other. Males have strongly pointed pectoral fins of triangular shape with a thickened second ray, ventral fins achieving an anus and considerable thickening of back. Moreover, a body conformation of a male is more gracile compared to that of a female. Females have pectoral fins of round shape, ventral fins non-achieving the anus. They usually achieve bigger size compared to males (Geldhauser, 1992; Kotusz, 1995; Drozd, present study – Figure 1).

To date, however, there is unsufficient evidence about achievement of sexual maturity in *M. fossilis*. Based on Bohl´s (1993) and our unpublished data, the secondary sex characteristics as well as formed gonads appear in the biggest males with total length ca. 13–14 cm already during the second year of life, in the age 1.5 years. Nevertheless, Bohl (1993) used for the artificial reproduction males stripped for the first time no earlier then in their third year of life. In related species *M. anguillicaudatus*, males and females spawn for the first time at the age of 1 and 2–3 years, respectively (Lei and Wang, 1990).

In nature, *M. fossilis* spawns from April to June (Grieb, 1937; Kryzanovskij, 1949; Kotlyarevskaja, 1967) depending on water temperature, usually from 13–14 °C (Kryzanovskij, 1949; Kotlyarevskaja, 1967). Weatherfish is a phytophilous species laying moderately sticky eggs (Geldhauser, 1992; Bohl, 1993) usually on submerse plants (Grieb, 1937; Kotlyarevskaja, 1967) or in their absence, on the root system of riparian vegetation (Klupp and Popp, 1992). The absolute female fecundity is over 100 thousand eggs (Susta, 1937; Bauch, 1953). Loaches are commonly multiple fractional spawners as shown by Suzuki (1983) and Bohlen (1999a) in *M. anguillicaudatus* and in spined loach *Cobitis taenia* (sensu L.), respectively. To date, we have no such information for *M. fossilis*.

A successful artificial reproduction of *M. fossilis* induced by means of gonadotropin contained in carp pituitary administered at doses ranging from 3 to 12 mg.kg$^{-1}$ body mass, b.m. but usually 5 mg.kg$^{-1}$ b.m., were firstly made by the Soviets, as it has been summarized by Kostomarova (1975), then by Geldhauser (1992), Bohl (1993), Kouril et al. (1996) and Adamkova-Stibranyiova et al. (1999). Experimentally evaluated female fecundity attributes according to Geldhauser (1992), Bohl (1993), Kouril et al. (1996) and Adamkova-Stibranyiova et al. (1999) were found ranging as followed: absolute stripping fecundity expressed by the total number of stripped eggs per female and stripping from $4 . 10^3$ to $20 . 10^3$ eggs with $8 . 10^3$ eggs as a mean, relative mass of stripped eggs expressed in percentage of body mass of the females prior to stripping from 20% to 27% with 25% as a mean and average wet mass of 1 egg from 0.9 mg to 1.1 mg.
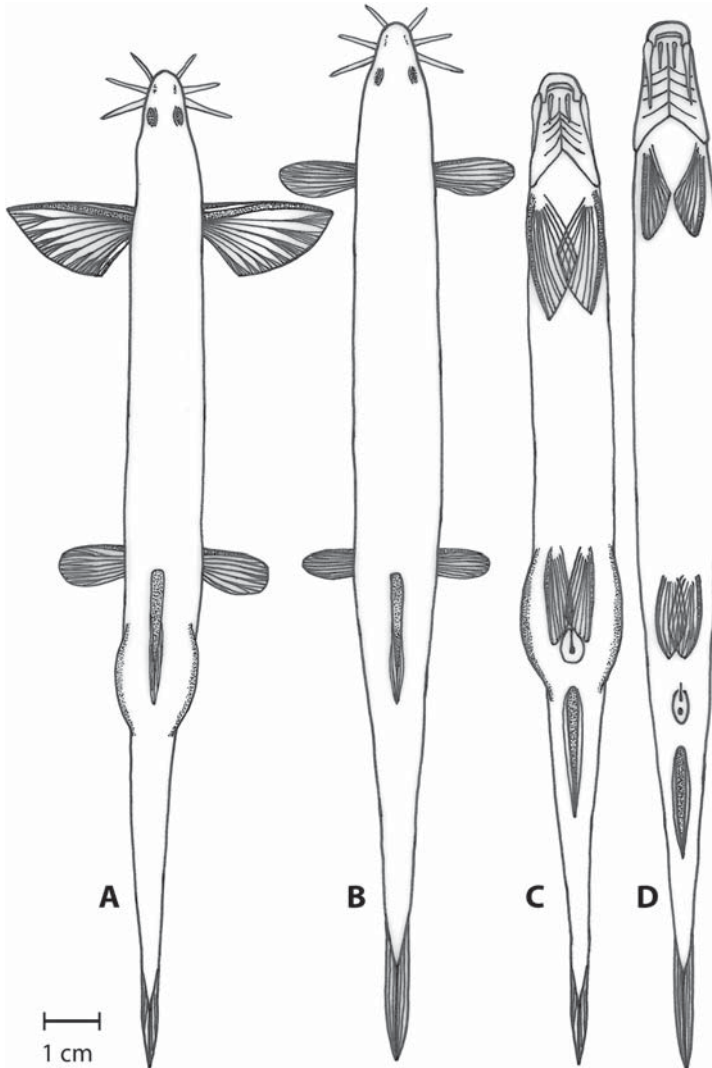
**Figure 1.** *Morphology of sexual dimorphism in M. fossilis. Male (A – dorsal view, C – ventral view) possesses the strongly pointed pectoral fins of triangular shape with a thickened second ray, ventral fins achieving an anus and considerable thickening of back. Female (B – dorsal view, D – ventral view) has the pectoral fins of round shape and ventral fins non-achieving the anus.*
*A schematic line drawing with highlighted secondary sex characteristics and omitted natural body pigmentation.*

## 1.6. LIFE HISTORY

Events which happen during the early life of fish, especially during their embryonal, larval and early juvenile periods are considered by many scientists to be of crucial or key importance in light of fluctuations of size and further existence of fish population in both freshwater and marine fish species (Chambers and Trippel, 1997).

Fish life history (ontogeny), defined as a set of morphological and physiological changes of an individual from egg activation to death (Kamler, 2002), may be influenced by many various endogenous factors conditioned by quality of broodstock and their gametes, as well as by exogenous factors of either biotic (predation, parasitism, diseases, food ability) or abiotic nature (temperature, oxygen concentration, pH, salinity, water current; Kamler, 2002).

## 1.6.1. The impact of temperature on fish

Temperature, however, is believed to be one of the most important factors mentioned above, affecting the life processes in fish in principal and various ways (Kamler, 1992), e.g. their sex determination (Conover and Kynard, 1981), metabolic rate, growth rate, diverse physiological functions (Blaxter, 1992), locomotor activity (Green and Fisher, 2004), development (Penaz et al., 1983; Kamler et al., 1998) or plasticity of morphometric characteristics (Stouracova et al., 1988; Penaz et al., 1989). In general, temperature has a major regulatory effect on physiology, physics, reproductive cycle and population dynamics of fish.

A specific temperature range within which a life of all members of particular fish population normally proceeds, is called range of tolerance or zone of thermal tolerance. Temperatures immediately below and above this range at which an individual survives for a certain period but is unable to complete a full life cycle, are considered as the temperatures within ranges of resistance. Temperatures lying outside of range of resistance towards lower or higher values, respectively, are believed to be lethal temperatures (Wieser, 1991).

In light of early fish ontogeny within the range of tolerance, subranges are distinguished corresponding to the optima for the diverse life processes (Kamler, 2002), such as growth, yolk mass utilization efficiency or survival [survival rate over 60% according to criteria of Kostomarova (1975), Penaz et al. (1983) and Ozernyuk et al. (1987)].

Fishes tolerating a narrow temperature range are denoted as stenothermic species. Those tolerating a wide temperature range are denominated as eurythermic ones. According to Wieser (1991), depending on whether the eurythermic fish species prefer cold- or warm temperature range, species are then distinguished either as cold-eurythermic (e.g. salmonids of the genus *Oncorhynchus* – see Kamler, 2002) or warm-eurythermic (few representatives of cyprinids such as the grass carp, *Ctenopharyngodon idella* or silver carp, *Hypophthalmichthys molitrix*; clariids as the North African catfish, *Clarias gariepinus*; or perciform fishes such as the Nile tilapia, *Oreochromis niloticus* – see Kamler, 2002). Fish species that prefer the middle temperature range are then denoted as mesothermic species (most cyprinids – see Kamler, 2002).

The extent and position of the zone of thermal tolerance of each particular species can vary in dependence on a set of genetically conditioned internal factors and on external factors, from which the acclimation (pretreatment) temperature is considered the most important one. A form of adaptation, which consists in increasing the thermal resistance up to the maximal value (reach of limit of the reaction standard of the species) with elevating acclimation temperature, is called resistance adaptation (Wieser, 1991). In context of the previous, the preferred temperatures (temperature range chosen by an individual if offered a choice) intraspecifically vary depending upon acclimation temperature, then upon time of the year (Cherry et al., 1975) or they vary during a single day (Reynolds et al., 1978). Process of resistance adaptation usually takes several days or weeks and involves series of large reorganization and readjustment of cellular structures and metabolic pathways (Wieser, 1991).

The study of effect of temperature on fish physiology is prevailingly focused on the investigation of impact temperature on locomotor activity, heart and muscle contraction frequency, oxygen

consumption and digestion (Wieser, 1991; Hunt von Herbing, 2002).

As summarized by Hunt von Herbing (2002), the temperature affects both directly the fish muscle physiology through its effect on the rates of enzymatic reactions and indirectly also the fish muscle physics due to its impact on water viscosity. How it seems to be, swimming activity reaches the minimal values at temperatures within the range of preferred temperatures for a particular species, which is naturally reflected in tendency of fish to maximally prolongate the time interval spent in these temperatures (Wieser, 1991).

The critical swimming speed, which markedly influences an ability to be or not to be predated (Wootton, 1990), is strongly affected by the reached developmental stage (Green and Fisher, 2004) and it is significantly rising with increasing temperature up to characteristic value above which it is usually declining again (Wieser, 1991). Oxygen consumption increases with the rising temperature and locomotor activity, most probably owing to the exponential relation of swimming speed and energy utilization efficiency. Closeness of the relation of energy turnover and temperature is significantly higher in the lower- compared to the upper temperature range (Wieser, 1991; Hunt von Herbing, 2002).

Besides a food intake ability, which influences an amount of ingested food, a quantity of energetic budget utilized by fish for common metabolic pathways, locomotion, growth or reproduction is affected by the ability to digest ingested food particles, which is also conditioned by the activity of digestive enzymes (Wieser, 1991; Hunt von Herbing, 2002).

Generally the types, secretion and activity of digestive enzymes involved in food digestion differ widely between species (Chakrabarti et al., 1995; Hidalgo et al., 1999). Intraspecifically, this varies depending upon developmental stage (Baragi and Lovell, 1986; Baglole et al., 1998; Chakrabarti et al., 2006; Faulk et al., 2007), individual fitness (Bolasina et al., 2006), diet (Papoutsoglou and Lyndon, 2006), and environmental factors. Depending upon fish species and type of digestive enzyme, particularly the season and lower temperature may lead to gradually declining or even to full suppression of digestive enzyme secretion and activity (Hidalgo et al., 1999; Kolkovski, 2001; Logothetis et al., 2001; Kofuji et al., 2005), including the decrease in the digestive contribution of live food organisms themselves (Dabrowski and Glogowski, 1977a, b). Although fish apparently ingest food particles, it may be resulted in total energy exhaustion and death of fish owing to retarded or fully stopped physiological processes as a consequence of long-term exposure to low temperatures which are out of species specific temperature range for normal proceeding. In contrast to the previous, an exposure to too high temperatures leads to heat coma or heat death as a result of process of protein denaturation (Wieser, 1991; Hunt von Herbing, 2002).

### 1.6.2. Crucial temperature-developmental theory

In fishes, however, their survival rate is considered a variable significantly affected by both the gained developmental stage and size. Embryos, i.e. individuals which are developing in eggs, eleuterembryos and/or larvae shortly after hatching represent ontogenetic stages which are the most sensitive to predation and fluctuation of environmental conditions and thus they suffer from the highest mortality rate (more than 99%; Chambers and Trippel, 1997). The temperature range for embryonic development is invariably narrower than those for juvenile and adult development (Wieser, 1991; Kamler, 1992).

Many of the recent studies focused on early development of fish arose to predict population dynamics and life requests of various species for the intents of fisheries management (Keckeis et al., 1996; Kamler et al., 1998; Klimogianni et al., 2004; Jordaan et al., 2006).

Consequently, understanding the temperature impact just on early life history of a fish is a crucial moment to understand the distribution and dynamics of fish egg and early developmental stages in

the nature, as well as for successful reproduction and high survival rate of early ontogenetic stages resulting in restoration and accruement of abundance of artificially made, managed and utilized fish populations (Kamler, 1992; Chambers and Trippel, 1997).

### 1.6.3. Early life history in weatherfish

Nearly all knowledge about *M. fossilis* ontogeny was mostly obtained by Soviet and later by Russian researchers. The first one, Grieb (1937) focused his study predominantly on development of additional respiratory organs of larvae, i.e. outer filamentous gills. Fundamental study of Kryzanovskij (1949) followed by a monographic chapter of Kostomarova (1975) had a cardinal importance for understanding the ontogeny of *M. fossilis*. Based on data of Soviet researchers only, Kostomarova collected everything known about weatherfish ontogeny, created a table of *M. fossilis* development, and suggested *M. fossilis* as a suitable model organism for embryological, biochemical, physiological, cytological and other studies. The reasons were as followed: short embryonic period, easy artificial spawning, absence of any problems with broodstock storage. However, we have to bear in mind to be aware in dealing or handling with data from the above literature, because of possible inaccuracies as a result of incubating eggs of unknown origin and age [data of Grieb (1937)] and unstable water conditions during egg incubation – in terms of temperature [data of Kryzanovskij (1949) and Geldhauser (1992)], respectively. Information about *M. fossilis* early life history is further supplemented by data originating from an artifical propagation and larvae rearing under artificial conditions made by Geldhauser (1992) and Kouril et al. (1996), and by implementation of information about early ontogeny of related species *M. anguillicaudatus* (Fujimoto et al., 2006).

### 1.7. PLOIDY LEVEL

Available evidence about chromosome number and/or ploidy level (the number of sets of chromosomes in cells) in weatherfish, *Misgurnus fossilis* (L.) provides an inconsistent or contradictory data pattern. Post (1965) reported the haploid chromosome number n = 24 indicating 2n around 50 for fishes from northern Germany. Recently, the most frequent situation are the findings of  Raicu and Taisescu (1972), Boroń (2000) and Ene and Suciu (2000) report on individuals with diploid chromosome number 2n = 100 in specimens of the Black Sea and Baltic Sea drainages (Raicu and Taisescu, 1972; Boroń, 2000; Ene and Suciu, 2000). However, Vujošević et al. (1983) described individuals with diploid chromosome number 2n = 50 from the Moravica River (Danube basin, Black Sea, Serbia). Finally, Palíková et al. (2007) in a genotoxicity study used, who studied chromosomal abberations in weatherfish embryos exposed to cyanobacterial extracts, as standards individuals with 100, but also with 50 chromosomes originating from a farmed weatherfish stock from Morava R. (Danube basin, Black Sea, Czech Republic). Individuals of *M. fossilis* with 100 chromosomes behave undoubtedly as biological diploids (Boroń, 2000), but they very likely arose via ancestral polyplodization event as could be deduced following suggestion of Ene and Suciu (2000) from deeper analysis of karyotype consisting of chromosome quadruplets.

Recent autopolyplodization events of *M. fossilis* may be hypothesized, because 25 quadrivalents, instead of 50 bivalents, are formed during mitosis as experimentally shown by Palíková et al. (2007).

Diploid chromosome numbers 2n = 50 were reported in other *Misgurnus* species *M. mizolepis* (Günter) by Ueno et al. (1985), Lee et al. (1987), *M. nikolskyi* (Vasiľeva) by Vasiľev and Vasiľeva (2008), *M. mohoity* (Dybowski) by Vasiľev and Vasiľeva (2008), but 2n = 48 or 49 was shown in *Paramisgurnus*

*dabryanus* (Dabry de Thiersant) by Li et al. (1983). However, a review of various forms of oriental weatherfish *Misgurnus anguillicaudatus* (Cantor) by Arai (2003) demonstrated existence of diploids (2n = 50), triploids (3n = 75) and tetraploids (4n = 100 chromosomes) in the wild, as well as of pentaploids and hexaploids (125 and 150 chromosomes, respectively) produced by chromosome set manipulation techniques.

## 1.8.   IMPORTANCE OF THE SPECIES

The floodplain river areas are commonly thought to enable and enhance fish recruitment by providing suitable fish spawning and nursery areas because of their habitat diversity, richness in shelters for protection from strong water current and predation, as well as in small food particles (Copp and Penaz, 1988; King et al., 2003). As a response to regulation of flow, as well as the entire river systems, floodplain pools and other off-channel water bodies gradually lost their previous ecological function, because of their isolation from the trunk stream (Bain et al., 1988; Ward and Stanford, 1995; Aarts et al., 2004).

Nevertheless, as Ph.D. thesis of Grift (2001) revealed, presently undertaken revitalizations of such localities within inundation areas contribute to successful restoration of the original riverine fish communities. As a consequence of the preceding, fishes such as barbel *Barbus barbus* (L.) or nase *Chondrostoma nasus* (L.) (Mann, 1996), are believed to comprise a very important indicator group for estimating river integrity (Karr, 1991).

As it is true in rheophilic species, *M. fossilis* is crucial among those fishes for explaining river inundation areas' importance in respect to successful fish reproduction, as well as to distribution and dynamics of early developmental stages (Ward, 1998) that present the key events for fish recruitment and population dynamics (Kamler, 1992; Schiemer et al., 2003).

## 1.9.   AIMS OF THE PRESENT PH.D. THESIS

To date, however, there is insufficient knowledge concerning the thermal limits of early life history in *M. fossilis* (Kryzanovskij, 1949; Kostomarova, 1975; Alexeeva and Ozernyuk, 1987). In order to predict wild population dynamics and life demands of *M. fossilis* for purposes of effective conservation management, one of the principal aims of the present Ph.D. thesis was as followed:

**To evaluate experimentally the sensitivity of embryonic and larval development in *M. fossilis* to temperature and to find its real thermal requirements during the period from egg activation to the onset of exogenous feeding.**

Moreover, presence of *M. fossilis* specimens with different diploid chromosome number (2n = 50 or 2n = 100) provides evidence on existence of at least two different evolutionary lineages (with distinct ploidy levels), or even separate species then there are presently recognized as *M. fossilis*, similarly as with discovery of cryptic clonal lineages in *M. anguillicaudatus* by Morishima et al. (2002, 2008). Using data on geographical distribution of haplotype richness of weatherfish (Bohlen et al., 2007; Mendel et al., 2008) that survived over glacial maxima in the main refuge within Danube basin and in putative additional refuges, the other crucial aim of the present Ph.D. thesis was the following:

**To elucidate the question which ploidy levels occur in natural *M. fossilis* population** inhabiting the floodplain area of the Lužnice River using cytogenetic methods.

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# CHAPTER 2

## TEMPERATURE-INDUCED PLASTICITY OF EARLY LIFE HISTORY
## IN WEATHERFISH *Misgurnus fossilis* (L.)

**2.1.** Effect of temperature on early life history in weatherfish *Misgurnus fossilis* (Linnaeus, 1758)

Drozd, B., Kouřil, J., Bláha, M., Hamáčková, J., 2009. Effect of temperature on early life history in weatherfish *Misgurnus fossilis* (Linnaeus, 1758). Knowledge and Management of Aquatic Ecosystems 392 (Article number: 04), 17 pp.

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**2.2.** Temperature-induced ontogenetic plasticity in weatherfish *Misgurnus fossilis* (Cypriniformes, Cobitidae) during embryonic and larval periods

Drozd, B., Bláha, M., Hamáčková, J., Kouřil, J., 2010. Temperature-induced ontogenetic plasticity in weatherfish *Misgurnus fossilis* (Cypriniformes, Cobitidae) during embryonic and larval periods. Journal of Fish Biology. (submitted)

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# Effect of temperature on early life history in weatherfish, *Misgurnus fossilis* (L. 1758)

B. Drozd[1], J. Kouril[1], M. Blaha[1], J. Hamackova[1]

## Abstract

**Key-words:**
*weatherfish,
ontogeny,
temperature,
mortality*

Effect of incubation temperature (range: 9–36 °C; interval: 3 °C) on artificially propagated weatherfish (*Misgurnus fossilis*) early ontogeny (during interval from egg fertilization to the finish of hatching) was investigated. Both, the amplitude of the incubation period (evaluated in four crucial moments), the total hatching period duration was inversely proportional to the incubation temperature and ranged from 17.5 days at 9 °C to 1.8 days at 24 °C (expressed at $H_{50}$) or from 137 hours at 9 °C to 9 hours at 24 °C, respectively. There were no influence of rising temperature on the total length of newly hatched larvae ($T_L = 4.23$–$4.67$ mm), in contrast to negative correlation with developmental stage (9–18 °C: stage 37; 21–24 °C: stage 36), *i.e.* the length might determine the age at hatching, rather than the age at hatching determines the hatching length. The thermal tolerance range in term of survival lies between 9 and 24 °C (the thermal optimum 15–24 °C, *i.e.* weatherfish is a warm-mesothermic species). Temperatures above 24 °C (in our study 27–36 °C) are considered the lethal temperatures already during embryonic period. It is highly recommended to distinguish an impact of suboptimal temperatures 9–12 °C on development during explored interval only, in contrast to possible other effect of these lower temperatures in context of the whole early ontogeny.

## RÉSUMÉ

Effets de la température sur les premiers stades de vie de la loche d'étang, *Misgurnus fossilis* (L. 1758)

**Mots-clés :**
*loche d'étang,
ontogénie,
température,
mortalité*

L'effet de la température d'incubation (gamme : 9–36 °C, intervalles : 3 °C) sur les premiers stades ontogéniques de loches d'étang (*Misgurnus fossilis*) (de la fécondation à la fin de l'éclosion) a été étudié. La durée de la période d'incubation (évaluée à quatre moments clés) et la durée de la période d'éclosion des œufs d'un lot sont inversement proportionnelles à la température d'incubation et s'étalent de 17,5 jours à 9 °C à 1,8 jour à 24 °C (pour l'indice $H_{50}$) et de 137 heures à 9 °C à 9 heures à 24 °C, respectivement. Il n'y a pas d'influence d'une élévation de température sur la longueur totale des larves à l'éclosion ($T_L = 4,23$–$4,67$ mm), alors qu'il y a une corrélation négative avec le stade de développement (9–18 °C : stade 37 ; 21–24 °C : stade 36), *i.e.* la longueur semble déterminer l'âge à l'éclosion, plutôt que l'âge à l'éclosion déterminerait la longueur à l'éclosion. La gamme

*(1) University of South Bohemia Ceske Budejovice, Research Institute of Fish Culture and Hydrobiology in Vodnany (RIFCH), Laboratory of reproduction and intensive aquaculture, Zatisi 728/II, 389 25 Vodnany, Czech Republic, borekdrozd@seznam.cz*

de tolérance thermique en terme de survie va de 9 à 24 °C (l'optimum thermique est entre 15–24 °C, *i.e.* la loche est une espèce mésothermique chaude). Les températures supérieures à 24 °C (dans notre étude 27–36 °C) sont considérées comme températures létales dès la période embryonnaire. Il est vivement conseillé de distinguer l'impact des températures suboptimales 9–12 °C sur le développement pendant l'expérimentation d'un autre effet possible de ces températures basses dans le contexte de tout le développement ontogénique précoce.

## INTRODUCTION

Weatherfish, *Misgurnus fossilis* (L. 1758), is a small freshwater fish inhabiting slowly flowing or stagnant freshwater habitats with heavy water vegetation over-grown and muddy bottom (mixed with detritus and dead vegetation) in the inundation area (especially the secondary arms, isolated backwaters and pools in the floodplain) (Meyer and Hinrichs, 2000; Pekarik *et al.*, 2008). It is spread through the whole Europe from the North France to the Western Russia (including the Donau and Volga river basin) except for Scandinavia, Mediterranean and the British Isles (Kottelat and Freyhof, 2007). In nature, weatherfish is spawning from April to June (Grieb, 1937; Kryzanovskij, 1949; Kotlyarevskaja, 1967) in dependence on water temperature (Kryzanovskij (1949) and Kotlyarevskaja (1967) states temperature 13–14 °C).

However, this species possesses the unique anatomical, morphological (outer filamentous gills in larvae – Grieb, 1937; Kryzanovskij, 1949; Kostomarova, 1975) and physiological adaptations (intestinal and skin breath – Park and Kim (1999) – *M. anguillicaudatus* (Cantor, 1842)), its density decreased rapidly through the whole Europe in the second part of the 20th century (Meyer and Hinrichs, 2000) due to the degraded water quality (impact of industry and agriculture), direct destruction and drying natural habitats (impact of water engineering), (leading to deficiencies in ecological integrity; Karr (1991)) just like in other freshwater species (Kamler *et al.*, 1998; Schiemer *et al.*, 2003). Nowadays its status in Europe is considered least concern (Kottelat and Freyhof, 2007), this species is listed under Annex II of the Council Directive 92/43/EEC (involved into Natura 2000 network) and in many Red lists of endangered fishes in the Europe.

Weatherfish belongs to the commercial unremarkable fish species, but also to the fishes crucial for explanation of floodplain areas importance (Ward, 1998) in respect to fish spawning, early ontogenetic stages distribution and dynamics (fishes are a very important indicator group for river integrity estimation – Karr (1991)).

Therefore a successful reproduction and a high level of survival of early ontogenetic stages are the key events for fish recruitment and fish population dynamics (Kamler, 1992; Schiemer *et al.*, 2003). Fish early life history can be affected by many biotic or abiotic factors such as temperature, concentration of dissolved oxygen (Kotlyarevskaja, 1967; Keckeis *et al.*, 1996; Bohlen, 2003), pH (Prokes *et al.*, 1998), water current (Schiemer *et al.*, 2003) or salinity (Bohlen, 1999a).

Temperature is considered one of the most important ones, affecting development and growth (Penaz *et al.*, 1983; Kamler *et al.*, 1998; Green and Fisher, 2004), morphometric features plasticity (Stouracova *et al.*, 1988; Penaz *et al.*, 1989), maximum swimming speed (Green and Fisher, 2004) or sex determination (Conover and Kynard, 1981). Many of the recent studies focused on early development of fishes arised to predict population dynamics and life requests of the various species for the intents of fisheries management (Keckeis *et al.*, 1996; Kamler *et al.*, 1998; Klimogianni *et al.*, 2004; Jordaan *et al.*, 2006). Therefore a comprehension of temperature effect on the early fish ontogeny is crucial in process of understanding of fish egg and early ontogenetic stages distribution, dynamics and mechanisms of adaptation (Klimogianni *et al.*, 2004) to the fluctuating environmental conditions in the floodplain area (Ward, 1998; Pekarik *et al.*, 2008).

Up to now, there is only limited knowledge concerning the temperature limits of weatherfish during early ontogeny (Kostomarova, 1975; Alexeeva and Ozernyuk, 1987; Zdanovich *et al.*,

2001). Therefore, the present study experimentally evaluates the thermal sensitivity of embryonic and larval development up to the finish of hatching in artificially propagated weatherfish in a wide temperature range.

# MATERIAL AND METHODS

## > BROODSTOCK AND EGG COLLECTION

Weatherfish (*M. fossilis*) broodstock (4 females: standard length = 223–241 mm, weight = 48–65 g; 3 males: standard length = 167–190 mm, weight = 20–30 g) were collected in April 2007 from a floodplain area of the Lužnice River (Czech Republic, South Bohemia). These fish were held in aquaria (volume = 30 L, temperature = 16–18 °C). In order to synchronize the spawning, the spermiation and ovulation were stimulated with carp pituitary (gonadotropin) injected intramuscularly at one dose of 5.00 mg·kg$^{-1}$ b.w. (body weight) for males, at two doses of 0.50 and 4.50 mg·kg$^{-1}$ b.w. (interval: 12 hours) for females. Ovulated oocytes were collected by hand-stripping and then fertilized with pooled milt (dry method) on 23rd April 2007. The broodfish were first anesthetized (clove oil, 0.07 mL·L$^{-1}$, 10 min) for safety manipulation during injection and hand-stripping. After spawning, all fish were released to the wild.

## > EGGS INCUBATION AND REARING CONDITIONS

Fertilized and unsticked eggs (stickiness was removed using half-fat milk diluted with water at a ratio 1:5 during 15 min) were incubated at 10 temperatures (range: 9–36 °C; interval: 3 °C). Temperatures were sustained by use of temperature-controlled refrigeration (or heating) system and measured by use of data loggers (RT-F53, Qi Analytical, Prague, Czech Republic) every 30 min in the range c. ± 0.5 °C. Eggs were put into the incubators (type I: glass beakers, volume = 250 mL; type II: transparent plastic boxes, volume = 2000 mL) placed in bath units (glass aquaria, water volume = 25 L) connected to temperature-controlling system. The temperature-acclimated reservoirs (volume = 2000 mL) served as a source for culture water replacement (oxygen saturated water) in each temperature unit.

In all units, eggs were divided in density of 50 eggs (incubator type I) and 1000 eggs (incubator type II). For each water temperature, two types of incubators were used – type I was used for estimation of mortality in triplicates, and type II for developmental sampling in duplicate.

Dissolved oxygen concentrations and pH were measured twice a day by handheld oxygen-pH meter (Oxi 315i, WTW GmbH) with additional regular monitoring of $NH_4^+$, $NO_3^-$, $NO_2^-$, chemical oxygen demand ($COD_{Mn}$) at 3-day intervals (in chemical laboratory). Photoperiod was maintained the same (12L:12D) in all temperatures (light intensity of 50–100 lx at the water surface). Summary of the main environmental parameters measured troughout the whole experiment is given in Table I. When it was possible, the experiments lasted for 19 days until hatching was finished, in all temperatures.

## > MORTALITY EVALUATION

At each water temperature (incubators type I), mortality of embryos (eggs) was recorded daily. White opaque eggs were identified as dead eggs and were siphoned off. A total of 105 observations for temperature 9–24 °C (interval: 0.5–6 hours), 6 observations for temperature 27–36 °C (interval: 0.5 hour) were made.

## > SAMPLING

Samples of at least 100 pieces of stripped unfertilized fresh eggs from each female for evaluation female fecundity features and egg characteristics (egg diameter and weight) were collected.

**Table I**
*Main physical-chemical water condition measured during M. fossilis egg incubation and free stages cultivation.*
*$cO_2$, concentration of dissolved oxygen (expressed in mg $O_2 \cdot L_{H_2O}^{-1}$/percent of oxygen saturation); $t_{water}$ water temperature (°C).*

Tableau I
Principaux paramètres physico-chimiques mesurés pendant l'incubation des œufs de *M. fossilis*, et les jeunes stades larvaires.
$cO_2$, concentration en oxygène dissous (exprimé en mg $O_2 \cdot L_{H_2O}^{-1}$/pourcentage de saturation en oxygène) ; $t_{water}$, température de l'eau (°C).

| Temperature | 9 °C | | | 12 °C | | | 15 °C | | | 18 °C | | | 21 °C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ |
| Minimum | 7.76 | 10.40/90.05 | 8.50 | 7.38 | 7.65/71.16 | 11.50 | 7.80 | 8.05/80.26 | 14.50 | 7.68 | 6.85/72.87 | 17.50 | 7.40 | 6.60/74.66 | 20.50 |
| Maximum | 8.20 | 12.50/108.23 | 9.50 | 8.19 | 11.05/102.79 | 12.50 | 8.17 | 10.35/103.19 | 15.50 | 8.52 | 9.45/100.53 | 18.50 | 8.31 | 8.70/98.42 | 21.50 |
| Mean | 7.98 | 11.29/97.75 | 8.90 | 7.91 | 9.51/88.46 | 12.10 | 8.00 | 9.12/90.93 | 14.90 | 8.01 | 8.40/89.36 | 17.85 | 8.00 | 7.60/85.97 | 21.20 |
| S.D. | 0.10 | 0.58/5.06 | 0.40 | 0.20 | 1.12/10.75 | 0.20 | 0.10 | 0.42/4.07 | 0.20 | 0.17 | 0.59/5.04 | 0.40 | 0.22 | 1.02/9.87 | 0.30 |

| Temperature | 24 °C | | | 27 °C | | | 30 °C | | | 33 °C | | | 36 °C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ | pH | $cO_2$ | $t_{water}$ |
| Minimum | 6.35 | 4.40/52.82 | 23.50 | 8.00 | 6.90/87.66 | 26.50 | 7.97 | 6.00/80.65 | 29.50 | 8.18 | 6.30/89.49 | 32.50 | 8.34 | 6.70/101.51 | 36.50 |
| Maximum | 8.29 | 8.55/102.64 | 24.50 | 8.26 | 7.60/96.57 | 27.50 | 8.36 | 7.20/96.78 | 30.50 | 8.42 | 6.90/98.01 | 33.00 | 8.41 | 6.70/101.51 | 37.00 |
| Mean | 8.00 | 7.10/85.23 | 23.90 | 8.10 | 7.23/91.87 | 26.80 | 8.16 | 6.50/87.37 | 29.80 | 8.30 | 6.60/93.75 | 32.80 | 8.38 | 6.70/101.51 | 36.00 |
| S.D. | 0.22 | 0.75/7.12 | 0.40 | 0.11 | 0.29/2.12 | 0.20 | 0.20 | 0.70/6.91 | 0.20 | 0.12 | 0.30/2.74 | 0.30 | 0.04 | 0.00/0.00 | 0.20 |

During early development, fish samples (usually 5–10, in the crucial points of hatching at least 30 eggs or free stages) were taken at regular 30 min (from egg fertilization (0 hour post-fertilization (hPF)) to 1 day post-fertilization (1 dPF), 2 hours (period: 2–3 dPF), 6 hours (period: 4–8 dPF), 12 hours (period : 9–19 dPF). Incubated eggs and newly hatched larvae (in total of at least 200 eggs or larvae per temperature) were preserved in 4% PFA (phosphate buffered paraformaldehyde solution) for 10–50 days. This period is recommended for next observation by Lusk and Pokorny (1964), when the changes of length (decrease 1–2%) and weight (increase 5–8%) characteristics reach the minimal values compared to situation in fresh unpreserved individuals.

### > DATA ANALYSES

Developmental stages were determined using criteria published in Kryzanovskij (1949) with more precise concept set out in Kostomarova (1975) and Fujimoto *et al.* (2006) (early ontogenetic stages in related species *M. anguillicaudatus*). Hatching of free swimming stages is considered the most important developmental treshold and the true onset of larval period (according to Kamler *et al.*, 1998). Within incubation period (time from egg fertilization to hatching of larvae) four serious moments (sample points) were distinguished : (a) start of hatching (*i.e.* the point of hatching of 5% individuals), (b) $H_{50}$ (*i.e.* the point of hatching of 50% individuals), (c) $H_{75}$ (*i.e.* the point of hatching of 75% individuals) and (d) finish of hatching (*i.e.* the point of hatching of 95% individuals). Time scale used for the development is presented as days post-fertilization (dPF).

Female fecundity features – the absolute stripping fecundity (total number of stripped eggs per female), the relative stripping fecundity (number of eggs per kg of female body mass (b. m.)) and the relative weight of stripped eggs (weight of stripped eggs/female weight prior to stripping) – were estimated using gravimetric method based on average wet weight of one egg (the last mentioned value was counted from the weight of mixed sample of 100 stripped unfertilized unpreserved eggs per one female). Average diameter of one egg was determined using the optical image analysis method (see below) of 200 stripped unfertilized unpreserved eggs (mixed sample coming from all females).

Cumulative mortality rate (from egg fertilization to finish of hatching) was calculated from the difference between initial numbers of eggs after fertilization and numbers of survived larvae after hatching, when possible (measurements at temperatures 27–34 °C finished earlier because of dying of all eggs before start of hatching).

Fixed individuals were examined under a binocular microscope (Olympus SZ 40, SZX9) and photographed using binocular microscope, fitted with a phototube and digital camera (Sony Progressive 3CCD and Olympus Camedia C5060UW). Digital images were then analysed (by video image analysis software – MicroImage version 3.0.1 for Windows) for total length ($L_T$) determination.

Data for the amplitude of the incubation period (assessed in four crucial points) and the total hatching period duration, were analysed by multiple regression method (test criterion F, correlation coefficient R, *P* value) to determination and graphic visualization (2D graphs method was used) of the relationship between observed parameters and temperature. One-way ANOVA (including Tukey HSD test in next step) was used for evaluation of possible significant difference in $L_T$ (measured at $H_{50}$) among constituent temperature groups. Programme Statistica 7.0 (StatSoft, Inc.) was used for data analysis. However, water temperatures 27, 30, 33 and 36 °C were excluded from further statistical analyses due to fatal effects in term of larvae survival (complete mortality) already during early embryonic period.

## RESULTS

Artificial stripping was successful in 100 percent hormonally stimulated females. The interval of latency (*i.e.* the period after second pituitary dose injection to egg release) varied
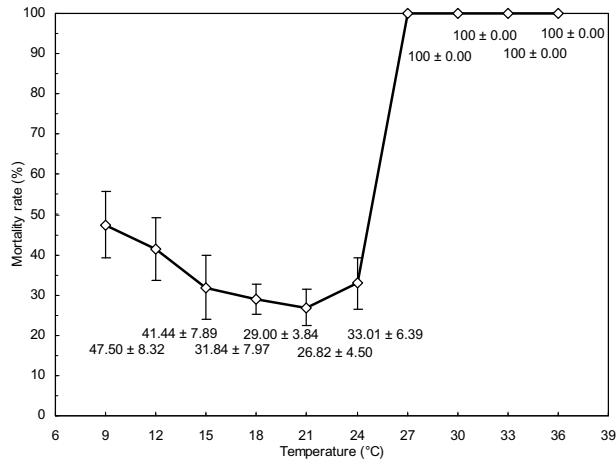
**Figure 1**

*Cumulative mortality rate (mean ± S.D.; expressed in percents) in* M. fossilis *between egg fertilization and finish of hatching (*i.e. *the point of hatching of 95% individuals) in relation to temperature (*n = *50 for each temperature).*

Figure 1

Taux cumulatif de mortalité (moyenne ± S.D. ; exprimés en pourcentage) chez *M. fossilis* (de la fertilisation des œufs à la fin de l'éclosion (*i.e.* point d'éclosion de 95 % des individus) en relation avec la température (*n* = 50 pour chaque température).

from 16.00 to 17.50 hours (16.50 ± 0.50, mean ± S.D.), in contrast to 33 percent achievement in hypophised males. Total number of stripped eggs (the absolute striping fecundity) ranged between 5800–7900 (6900 ± 830) eggs per female. The relative stripping fecundity (number of eggs/kg b. m.) varied from 88 to 135 (121.60 ± 19.50) thousand eggs per female. The relative weight of stripped eggs formed 8.40–13.00 (10.70 ± 1.70) percent of female weight prior to stripping. The egg wet weight ranged between 0.76–0.96 mg (0.88 ± 0.08; $N = 400$), the egg diameter varied from 1.37 to 1.66 mm (1.42 ± 0.11; $N = 200$).

The accumulated mortality rate (mean ± S.D.) between egg fertilization and finish of hatching (*i.e.* the point of hatching of 95% individuals) reached value 47.50 ± 8.32% (at temperature 9 °C), 41.44 ± 7.89% (12 °C), 31.84 ± 7.97% (15 °C), 29.00 ± 3.84% (18 °C), 26.82 ± 4.50% (21 °C), 33.01 ± 6.39% (24 °C). At temperature 27–36 °C mortality increased up to 100% dead eggs, when all incubated weatherfish eggs were dying after 8.50 (27 °C), 8.00 (30 °C), 3.75 (33 °C) and 1.00 (36 °C) hours post-fertilization (Figure 1).

The amplitude of the incubation period (time from egg fertilization to hatch of larvae) decreased according to rising temperature. Mean time to reach all four sample points (see Material and Methods) was inversely proportional to temperature, *i.e.* ranged from c. 13 days at 9 °C to 1.60 days at 24 °C (start of hatching), from c. 17.50 days at 9 °C to 1.80 days at 24 °C ($H_{50}$), from c. 18 days at 9 °C to 1.90 days at 24 °C ($H_{75}$) and from c. 18.50 days at 9 °C to 2 days at 24 °C (finish of hatching) (Table II). Values of the start of hatching showed the negative exponential relationship with incubation temperature (Figure 2) (evaluated by multiple regression method; 2D graphs) described by formula: $y = 40.1354*e^{(-0.1402*x)}$ ($F_{(1, 178)} = 148.89$, $P < 0.01$, R = 0.92) as well as $H_{50}$ ($y = 50.7341*e^{(-0.1541*x)}$; $F_{(1, 178)} = 129.10$, $P < 0.01$, R = 0.90), $H_{75}$ ($y = 57.3162*e^{(-0.1508*x)}$; $F_{(1, 178)} = 130.38$, $P < 0.01$, R = 0.91), the finish of hatching ($y = 57.2396*e^{(-0.1474*x)}$; $F_{(1, 178)} = 130.21$, $P < 0.01$, R = 0.91).

Temporal ontogeny prolongation in dependence on temperature is also appreciable in the total hatching period duration (*i.e.* interval from start to finish of hatching), which is decreasing with rising temperature, from c. 137.00 hours at 9 °C to 9.00 hours at 24 °C (Table II),

**Table II**

*The amplitude of the incubation period (time from egg fertilization to hatch of larvae) evaluated in four critical points and total hatching period duration (i.e. interval from start to finish of hatching) (mean ± S.D.) in* M. fossilis.

*T, temperature; hPF, hourpost-fertilization (first line); dPF, day post-fertilization (second line); h, hour; N, number of observations; start, start of hatching (i.e. the point of hatching of 5% individuals); H$_{50}$, point of hatching of 50% individuals; H$_{75}$, point of hatching of 75% individuals; finish, finish of hatching (i.e. the point of hatching of 95% individuals).*

Tableau II
Amplitude de la période d'incubation (de la fertilisation à l'éclosion des larves) évaluée à quatre moments critiques et durée totale de la période d'incubation (*i.e.* intervalle du début à la fin des éclosions) (moyenne ± S.D.) chez *M. fossilis*.
T, température ; hPF, heures post-fertilisation (première ligne) ; dPF, jours post-fertilisation (seconde ligne) ; h, heure ; *N*, nombre d'observations ; start, début d'éclosion (*i.e.* le point d' éclosion de 5 % des individus) ; H$_{50}$, point d'éclosion de 50 % des individus ; H$_{75}$, point d'éclosion de 75 % des individus ; finish, fin de l'éclosion (*i.e.* le point d'éclosion de 95 % des individus).

| T | | Hatching (hPF/dPF) | | | | | | | | Hatching period | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | start | | H$_{50}$ | | H$_{75}$ | | finish | | duration (h) | | |
| | | Mean | S.D. | Mean | S.D. | Mean | S.D. | Mean | S.D. | N | Mean | S.D. |
| **9 °C** | 30 | 309.15 | 7.17 | 422.95 | 7.98 | 435.15 | 6.41 | 446.75 | 1.64 | 30 | 136.80 | 5.67 |
| | | 12.88 | 0.30 | 17.62 | 0.33 | 18.13 | 0.27 | 18.61 | 0.07 | | | |
| **12 °C** | 30 | 170.65 | 6.13 | 191.95 | 5.41 | 198.75 | 2.28 | 205.70 | 6.37 | 30 | 34.25 | 3.23 |
| | | 7.11 | 0.26 | 8.00 | 0.23 | 8.28 | 0.10 | 8.57 | 0.27 | | | |
| **15 °C** | 30 | 110.55 | 3.82 | 129.15 | 3.92 | 132.95 | 2.77 | 140.05 | 2.35 | 30 | 29.10 | 1.27 |
| | | 4.61 | 0.16 | 5.38 | 0.16 | 5.54 | 0.12 | 5.84 | 0.10 | | | |
| **18 °C** | 30 | 71.35 | 2.33 | 77.55 | 2.29 | 81.15 | 1.46 | 87.15 | 1.86 | 30 | 16.80 | 1.60 |
| | | 2.97 | 0.10 | 3.23 | 0.10 | 3.38 | 0.06 | 3.63 | 0.08 | | | |
| **21 °C** | 30 | 46.75 | 2.59 | 52.60 | 0.86 | 53.75 | 1.05 | 57.65 | 1.59 | 30 | 10.90 | 0.80 |
| | | 1.95 | 0.11 | 2.19 | 0.04 | 2.24 | 0.04 | 2.40 | 0.07 | | | |
| **24 °C** | 30 | 38.65 | 0.86 | 42.60 | 0.62 | 44.35 | 0.72 | 47.65 | 0.78 | 30 | 9.00 | 0.69 |
| | | 1.61 | 0.04 | 1.78 | 0.03 | 1.85 | 0.03 | 1.99 | 0.03 | | | |

is followed by negative exponential relationship with temperature (evaluated by multiple regression method; 2D graphs) described by formula: $y = 390.9970*e^{(-0.1677*x)}$ (F (1, 178) = 86.73, P < 0.01, R = 0.81) (Figure 3).

The total length of newly hatched larvae (evaluated during H$_{50}$) varied from 4.23 ± 0.24 mm to 4.67 ± 0.22 mm (mean ± S.D.) (Table III). The significant difference among temperatures (ANOVA F(5,75) = 6.24, P < 0.01) was found. The total length did not significantly differ in larvae incubated at 9, 15, 18, 21, 24 °C compared to situation at 12 °C (Tukey HSD test, P < 0.05) (Table III).

The ontogenetic stage at hatching tended to decrease with rising temperature. Eggs incubated at temperatures between 21 and 24 °C hatched at Kostomarova's (1975) stage 36 (*i.e.* stage with first visible otholits in othic capsule, head slightly separated from the yolk sac, 10–13 caudal somites, still curved caudal part), (or the 44-somite stage of *M. anguillicaudatus* – Fujimoto *et al.* (2006)) and those incubated at 9–18 °C, showed the traits of the stage 37 (*i.e.* stage with first visible germ of pectoral fin, head noticeably separated from yolk sac, 17 caudal somites, straight caudal part), (or the 50-somite stage of *M. anguillicaudatus* – Fujimoto *et al.* (2006)) at hatching.
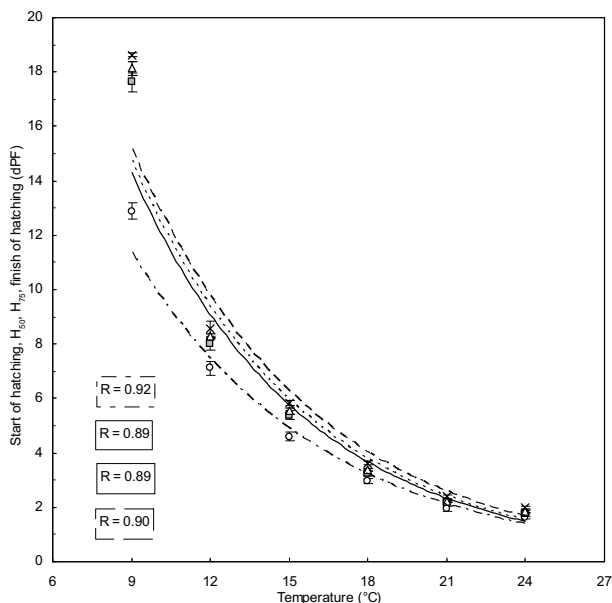
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**Figure 2**

*Relation of time until start of hatching (*i.e. *the point of hatching of 5% individuals), $H_{50}$ (*i.e. *the point of hatching of 50% individuals), $H_{75}$ (*i.e. *the point of hatching of 75% individuals), finish of hatching (*i.e. *the point of hatching of 95% individuals) and temperature (mean ± S.D.) in* M. fossilis *(without lethal temperature 27, 30, 33, 36 °C) (fitted by exponential function).*
*dPF, days post-fertilization; ·--·○·--·, start of hatching; ——□——, $H_{50}$; ····Δ····, $H_{75}$; ---×---, finish of hatching; R denotes correlation coefficient.*

Figure 2
Relation entre la durée d'incubation jusqu'au début de l'éclosion (*i.e.* point d'éclosion de 5 % des individus), $H_{50}$ (*i.e.* point d'éclosion de 50 % des individus), $H_{75}$ (*i.e.* point d'éclosion de 75 % des individus), et la durée d'incubation jusqu'à la fin de l'éclosion (*i.e.* point d'éclosion de 95 % des individus) et la température (moyenne ± S.D.) chez *M. fossilis* (sans les températures létales 27, 30, 33, 36 °C) (ajustement à une courbe exponentielle).
dPF, jours post-fertilisation ; ·--·○·--·, début d'éclosion ; –□– , $H_{50}$ ; ····Δ····, $H_{75}$ ; ---×---, fin d'éclosion ; R est le coefficient de corrélation.

## DISCUSSION

The absolute stripping fecundity (approximately 6.90 thousand eggs on average) of weatherfish female evaluated in our study reached the same figure as Bohl (1993), but 20% or 50% or even 60% lower value compared to Geldhauser (1992), Kouril *et al.* (1996) or Adamkova-Stibranyiova *et al*. (1999) respectively. The conformable situation as in previous parameter was recorded in the relative stripping fecundity (121.60 thousand eggs per kg b. m. on average) and the relative weight of stripped eggs (10.70% on average) too, when our data averaged the half value compared to Geldhauser (1992), Kouril *et al.* (1996) and Adamkova-Stibranyiova *et al*. (1999). The reason for this discrepancy might be an origin of the fish. In our study, we used the adults from the wild (they spent only 14 days before stripping in captivity). In comparison to the authors cited above who used the broodstock reared the whole season in condition of intensive (or extensive) pond culture, *i.e.* in condition
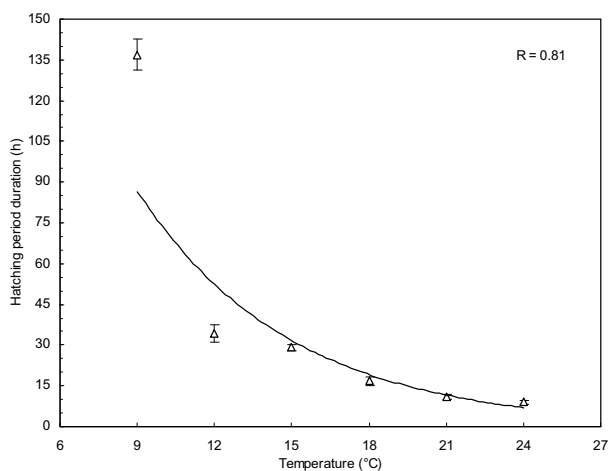
**Figure 3**
*Effect of temperature on the hatching period duration (*i.e. *interval from start to finish of hatching) (mean ± S.D.) in* M. fossilis *(without lethal temperature 27, 30, 33, 36 °C) (fitted by exponential function).*
*h, hours; R denotes correlation coefficient.*

Figure 3
Effet de la température sur la durée de la période d'éclosion (*i.e.* intervalle de temps entre le début et la fin de l'éclosion) (moyenne ± S.D.) chez *M. fossilis* (sans les températures létales 27, 30, 33, 36 °C) (ajustement à une courbe exponentielle).
h, heures ; R est le coefficient de corrélation.

**Table III**
*Total length of* M. fossilis *larvae (mean ± S.D.) after hatching (evaluated in $H_{50}$). Groups with the same superscript (a, b) do not significantly differ (Tukey HSD test,* P *< 0.05).*
$L_T$*, total body length;* N*, number of observations;* $H_{50}$*, point of hatching of 50% individuals.*

Tableau III
Longueur totale des larves de *M. fossilis* (moyenne ± S.D.) à l'éclosion (évaluée à $H_{50}$). Les groupes avec la même lettre (a, b) ne diffèrent pas significativement (Tukey HSD test, *P* < 0.05).
$L_T$, longueur totale ; *N*, nombre d'observations ; $H_{50}$, point d'éclosion de 50 % des individus.

| Temperature | $L_T$ (mm) [$H_{50}$] | | |
|---|---|---|---|
| | *N* | Mean | S.D. |
| **9 °C** | 30 | 4.31[a] | 0.15 |
| **12 °C** | 30 | 4.67[b] | 0.24 |
| **15 °C** | 30 | 4.29[a] | 0.23 |
| **18 °C** | 30 | 4.29[a] | 0.24 |
| **21 °C** | 30 | 4.23[a] | 0.24 |
| **24 °C** | 30 | 4.30[a] | 0.18 |

with relative sufficiency of food resources and stable water temperature and chemistry. In fact, we could obtain just one portion of eggs (based on information in related species *M. anguillicaudatus* reached by Suzuki (1983) under laboratory condition – data about fraction spawning in *M. fossilis* still absent). Fish species inhabiting the environment with often unfavourable conditions (high ammonium, sulphate concentration), frequent temperature, pH and water level disturbances (weatherfish is a typical representative) are often the multiple spawners (in order to extend reproductive period and to raise a probability of reproduction success), as described Bohlen (1999b) in the related species – spined loach (*Cobitis taenia – sensu* L. 1758) under laboratory conditions too.

In general, the absolute stripping fecundity (and the other fish female fecundity features derived from this parameter) should be considered the approximate parameter only (because of inaccuracies), highly dependent on fish attributes (age, size, health condition) reviewed by Kamler (2005), natural and artificial influence during pre-spawning (temperature, application of hormonal preparation and their dose) and spawning period (including stripping experience) (summarized by Kamler, 1992). However, in the endangered fish species such as weatherfish (the endangered and protected species in the whole Europe – see Introduction) this method is only one possible way to estimation of these data (dealing with fecundity) without need to kill the adult fish.

Both, the value of average wet egg weight (0.88 mg on average) and diameter (1.42 mm on average) obtained in our study, were significantly lower compared to the figures showed by Kouril *et al.* (1996), Adamkova-Stibranyiova *et al.* (1999) (our data are c. 10–15% lower) in case of egg wet weight and by Kryzanovskij (1949), Kostomarova (1975) (our data are c. 13–23% lower) in case of egg diameter. Nevertheless, the value of average egg diameter (e.d.) in weatherfish (reached during our experiment) is higher compared to the related "loach" species – *Misgurnus anguillicaudatus* (e.d. = 0.72–0.84 mm; Zheng, 1985), *M. mizolepis* (Günther, 1888) (e.d. = 1.10 mm; Kim *et al*., 1987), *Cobitis taenia* or *C. bilineata* (e.d. = 1.14 ± 0.07 mm or 1.21 ± 0.09 mm respectively; Bohlen, 1998).

However, we have to bear in mind a fact, that the average egg diameter and weight seems to be considerably influenced by yolk mass volume and dependent on the female attributes (Pepin *et al*., 1997; Marteinsdottir and Steinarsson, 1998), vary interseasonally (as well as during one individual season) and intraspecifically (between population), (Kamler, 1992; Marteinsdottir and Steinarsson, 1998) too. Therefore it is very difficult to make a serious comparison or deep analysis between our data and the cited ones, due to the absence of data regarding female condition (especially age, size, spawning experience and fecundity) in cited literature.

Both, the amplitude of the incubation period (evaluated in all four crucial moments mentioned above – see chapter Material and Methods) and the total hatching period duration were inversely proportional to the incubation temperature, in accordance to results of Penaz *et al.* (1983) (in common carp), Ojanguren and Braña (2003) (in brown trout), Klimogianni *et al*. (2004) (in common pandora) or Kamiński *et al.* (2006) (in lake minnow), *i.e.* the amplitude of the incubation period (or the total hatching period duration) at minimal temperature generally reached approximately nine times (or fifteen times, respectively) the value at maximal temperature.

Values of the incubation period amplitude (in temperature range 18–24 °C) evaluated in our study, reached the similar level (time thermal-dependent range) presented by Kouril *et al.* (1996) (at 18 °C), Kostomarova (1975) (at 21.50 °C) in weatherfish or Suzuki (1953) (19–21 °C), Watanabe *et al.* (1948) (20–21 °C), Fujimoto *et al*. (2006) (20 °C) in related species, oriental weatherfish (*M. anguillicaudatus*).

In contrast, Grieb (1937), Geldhauser (1992) in weatherfish or Zheng (1985) in oriental weatherfish present noticeably different figures compared to our findings. We probably have to look for a reason of this discrepancy either in an unknown origin of incubated eggs (Grieb, 1937) or in unstable water conditions during incubation (Kryzanovskij, 1949; Zheng, 1985; Geldhauser, 1992). Grieb (1937) used for his observation fertilized eggs from the wild (unknown age), subsequently incubated at temperature 15 °C in laboratory (but value

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*Temperature-induced plasticity of early life history in weatherfish Misgurnus fossilis (L.)*

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***Figure 4***
*Comparison of the amplitude of the hatching period (in hours post-fertilization) reached in our study (evaluated in points: start of hatching, H₅₀, finish of hatching) with data known from literature in relation to temperature.*
*White rectangle black framed, our data (in Misgurnus fossilis); grey rectangle, data from literature (in Misgurnus fossilis); white rectangle with grey letters, data from literature (in Misgurnus anguillicaudatus); start (of hatching), point of hatching of 5% individuals; H₅₀, point of hatching of 50% individuals; finish (of hatching), point of hatching of 95% individuals.*
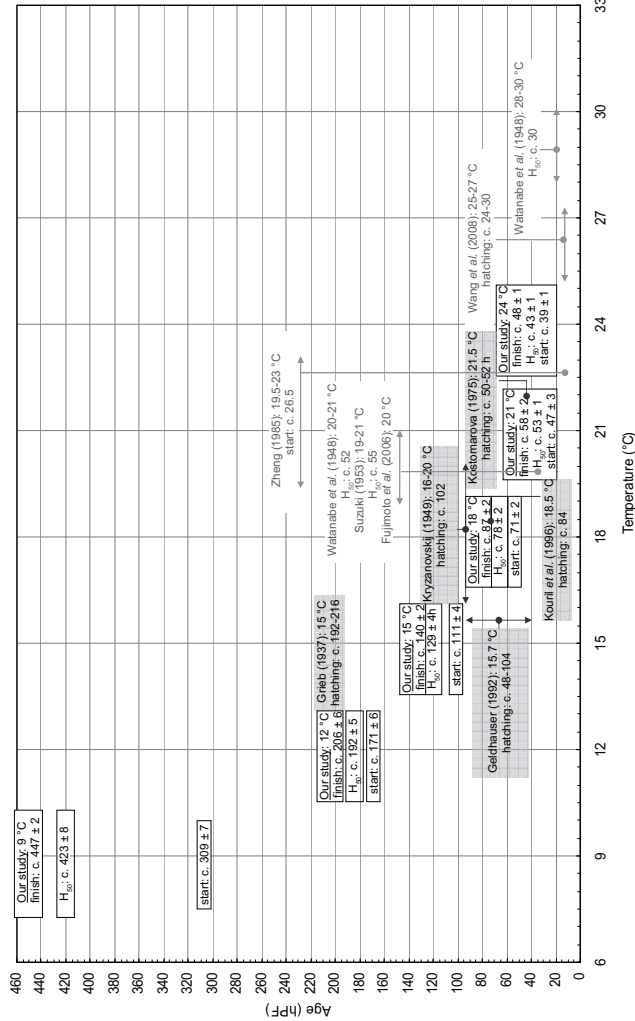
Figure 4
Comparaison de l'amplitude de la période d'éclosion (en heures après la fertilisation) obtenue dans notre étude (évaluée aux points : début d'éclosion, H₅₀, fin de l'éclosion) avec les données de la littérature en rapport avec la température.
Rectangle blanc à bordure noire, nos données (chez *Misgurnus fossilis*) ; rectangle gris, données de la littérature (chez *Misgurnus fossilis*) ; rectangle blanc avec lettres grises, données de la littérature (chez *Misgurnus anguillicaudatus*) ; start (début d'éclosion), point d'éclosion de 5 % des individus ; H₅₀, point d'éclosion de 50 % des individus ; finish (fin d'éclosion), point d'éclosion de 95 % des individus.

of the incubation period amplitude shown by Grieb (1937) presents a typical situation at 12 °C, in our study). Water temperature fluctuated in range 16–20 °C (Kryzanovskij, 1949), 19.50–23 °C Zheng (1985) or 15–16.90 °C (Geldhauser, 1992). Values of the incubation period amplitude shown by Kryzanovskij (1949), Zheng (1985) or Geldhauser (1992) present situation of fluctuation of water temperature in range 15–18 °C, around 24 °C or 15–21 °C respectively, in our study (value presented by Zheng (1985) probably represents data for hatching of larvae at earlier ontogenetic stage – but the author does not refer a developmental stage of newly hatched larvae). A summarized comparison of the hatching period amplitude reached in our study with data known from literature (in relation to temperature) is given in Figure 4 (values presented by Watanabe *et al*. (1948) and Wang *et al*. (2008) serve as the reference figures for hatching in temperature range 25–30 °C in *M. anguillicaudatus* – we did not observe hatching in weatherfish at temperatures above 24 °C (due to lethal impact – see Discussion below)).

Size of newly hatched larvae seems to be considerably influenced by parental attributes (Wootton, 1990; Panagiotaki and Geffen, 1992; Kamler, 2005), or water conditions (Keckeis *et al*., 1996; Prokes *et al*., 1998; Schiemer *et al*., 2003). We observed a significant decrease of the total length of newly hatched larvae (our data are c. 15% lower on average) compared to the figures presented by Grieb (1937), Kryzanovskij (1949), Kotlyarevskaja (1967) or Kostomarova (1975), probably caused by smaller egg size (observed during stripping – see Discussion above). According to Kotlyarevskaja (1967), a ground of this variance might be found in oxygen level (total length of freshly hatched weatherfish larvae reaches c. 5 mm in treatment with oxygen concentration 6.00–8.50 mg $O_2 \cdot L^{-1}$, or c. 4 mm in treatment with oxygen concentration 2.40–4.00 mg $O_2 \cdot L^{-1}$ respectively), but in our study, a concentration of dissolved oxygen overreached on average value 7 mg $O_2 \cdot L^{-1}$ (c. 90% of oxygen saturation) over all temperatures. Nevertheless, the size of larvae after hatching in weatherfish (reached in our study) generally represents double of the length presented by Zheng (1985) (c. 1.95–2.40 mm) or Fujimoto *et al.* (2006) (c. 2.60 mm on average) in newly hatched larvae of the related loach, *M. anguillicaudatus*.

Generally, preceding studies dealing with the effect of temperature on hatching size, have offered contradictory conclusions. The most common situation is an inverse correlation of the larval size at hatch and temperature (higher temperature produces smaller larvae; hatching is an age-related rather than size-related event) (Penaz *et al*., 1983; Ojanguren and Braña, 2003; Jordaan *et al.*, 2006).

In contrast to previous opinions, Alderdice and Forrester (1974) (in flathead sole) or Pepin *et al*. (1997) (in Atlantic cod) described a decrease of newly hatched larvae size with declining temperature (in latter, this situation might be considered the impact of increased metabolic requirements at temperatures close to the lower boundary of its thermal tolerance range).

However, our results do not suggest any correlation (neither positive nor negative) of the size of newly hatched larvae and temperature (in wide temperature range 9–24 °C), *i.e.* the total length of larvae at hatch do not significantly differ in dependence on temperature, according to situation observed by Blaxter and Hempel (1963) in herring or Jordaan *et al*. (2006) in Atlantic cod (batch 2). The significant difference of $L_T$ at 12 °C compared to other temperatures might be probably affected either by real impact of low temperature leading to bigger larvae, or more probably by collector's mistake, who selectively chose the larvae with bigger size (significant difference ($P < 0.05$) between 12 °C and other temperatures is only c. 0.30 mm on average).

In addition, our results suggested that the length might determine the age at hatching, rather than the age at hatching determines the hatching length. Therefore it has been assumed that embryonic growth (together with temperature) probably determines the time of hatching in *M. fossilis* (and therefore growth within eggs is most likely unequal in this species) at least within the temperature range that we used.

According to Yamagami (1988), the hatching event is only loosely linked to the developmental stage in many fish species. A developmental stage reached by weatherfish

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at hatching is negatively correlated with temperature (violation of the equiproportional rule), in accordance with results presented by Penaz *et al.* (1983) (in common carp) or Ojanguren and Braña (2003) (in brown trout). It means, eggs cultivated at temperature 21 and 24 °C hatch at the earlier ontogenetic stage (stage 36 or the 44-somite stage, respectively) compared to the stage 37 (the 50-somite stage, respectively) at temperature 9–18 °C (more detailed ontogenetic staging for finer distinguishing of particular ontogenetic stages, than in Kostomarova (1975) or Fujimoto *et al*. (2006), is not available). Variation of the reached stage at hatching (in our study) should not be caused by oxygen condition (concentration of dissolved oxygen was on average over 7 mg $O_2 \cdot L^{-1}$ (c. 90% of oxygen saturation) in all temperatures), compared to Kotlyarevskaja (1967) (stage 37 hatched in oxygen concentration 6.00–8.50 mg $O_2 \cdot L^{-1}$; stage 36 in oxygen concentration 2.40–4.00 mg $O_2 \cdot L^{-1}$, respectively).

However, correlation (positive or negative) of the developmental stage and the larval size at hatch (except at 12 °C – see comments above) was not found, in contrast to suggestion (positive correlation of developmental stage and size) of Penaz *et al*. (1983) (in common carp), Penaz *et al*. (1989) (in tench) or Ojanguren and Braña (2003) (in brown trout).

In term of environmental implications, a ground of hatching of weatherfish at almost the same size and developmental stage, practically non-affected by temperature (based on our data), might be explained as a set of adaptations for survival and successful reproduction in the wild. An outcome represents a functional compromise between ontogenetic and behavioural traits conditioned by endogenous and exogenous (abiotic and biotic) factors leading to a synergistic influence on fish populations and their dynamics.

In energetic aspect, larvae hatching at lower temperatures (at 9 and 12 °C) could not reach higher size due to a longer time spent in egg, leading to hatch of larvae with a low energetic source stored in a yolk sac (larvae hatch close to complete yolk sac depletion followed by starvation conducing to dead, soon) according to Kamler (1992).

Weatherfish inhabits environment with often unfavourable, all the time changing conditions (see Discussion – female fecundity) and puts sticky eggs over underwater vegetation (Grieb, 1937; Kryzanovskij, 1949). Weatherfish larvae after hatching hang fast-stuck to plants by head over several days (Grieb, 1937; Kryzanovskij, 1949; Kostomarova, 1975). Therefore, freshly hatched larvae (larvae incubated at higher temperature – in our study at 15–24 °C) in case of hatch at lower ontogenetic stage (according to a common opinion that higher temperature produces smaller larvae at the lower developmental stage – see above) may risk their soon death due to staying in condition of almost no oxygen, with low temperature and high concentration of amonium, sulphate or marsh gas (organs of an additional breething such as outer filamentous gills, segmental blood vessels in fin-fold or intestinal respiration occur even during stage 38, stage 39 or in adult fish, respectively – Kryzanovskij, 1949; Kostomarova, 1975; Park and Kim, 1999: *M. anguillicaudatus*). According to Kotlyarevskaja (1967), weatherfish larvae have to actively seek for suitable environmental conditions and feed by climbing over aquatic vegetation.

Biotic factors (especially interspecific food competition and predation) can be as important as egg size (see Discussion above) or abiotic environmental conditions (see above) in term of governing of size at hatch in weatherfish larvae (and reaching developmental stage too).

Larval size at hatch determines the initial food size by exogenous nutrition start (*i.e.* longer larvae at higher developmental stage have a chance to win in interspecific food competition) and together with egg size defines the predator size which is able to utilize these early ontogenetic stages (*i.e.* an earlier ontogenetic stage with bigger yolk sac might be more vulnerable to predation) (Wootton, 1990). The ability of larvae to react to assumed predation danger, maximum and mean escape speed increase after hatching till complete yolk sac depletion in dependence on temperature (Green and Fisher, 2004). In addition, predation risk may be notably scaled down during several days after hatching due to speedy growth and variation in behaviour related to ontogeny (Eeton and DiDomenico, 1986; Webb and Weihs, 1986).

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The accumulated mortality rate between egg fertilization and finish of hatching (*i.e.* the point of hatching of 95% individuals) varied from 26.80 to 100% in dependence on incubation temperature (in addition survival reached value 0.00–73.20% during the same period). Consequently, the thermal tolerance range for the early ontogeny (*i.e.* the period from egg fertilization up to finish of hatching) in *M. fossilis* lies between 9 °C and 24 °C.

Temperatures 15–24 °C are considered the range of the thermal optimum for weatherfish, (survival over 60% of eggs alive is considered the limit for optimum temperature classification by Kostomarova (1975) in weatherfish, Penaz *et al.* (1983) in common carp and Ozernyuk *et al.* (1987) in some cyprinids) in our study. These temperatures are also in accordance to the temperatures observed during spawning of weatherfish in the wild (Grieb, 1937; Kryzanovskij, 1949), as well as to the optimal range obtained under laboratory conditions (Kostomarova (1975) presents range (13)14–(20)24 °C; Alexeeva and Ozernyuk (1987) and Zdanovich *et al*. (2001) range 14–22 °C, respectively). Within the optimal thermal range (concretely at temperature 18 °C), we observed an apparent difference in mortality level (our data reached value 29%) compared to cited data (further mentioned), *i.e.* 10–15% lower value in comparison to Kouril *et al.* (1996) (mortality reached 40–50%), but c. 10% higher figure in comparison to Bohl (1993) (mortality at level 80%, presented by Bohl (1993) is probably caused by female over-maturation during end of June).

Nevertheless, we have to bear in mind a necessity to specify a term "optimal temperature". According to general opinions (summarized in Kamler (1992) or Pavlov (2007)), the optimal temperature range for embryonic/larval stages survival may not be the same, compared to the range for optimal growth or effectivness of food utilization (which we have not investigated in present study).

Relatively acute boundary in mortality of incubated eggs was observed between temperature 24 and 27 °C (accumulated mortality rate rapidly increased from above-average value (33%) up to 100%), when all weatherfish eggs were dying up to several hours post-fertilization. Consequently, temperatures above 24 °C (in our study temperature in range 27–36 °C) are considered the lethal temperatures due to the live-inhibiting effect already during embryonic period.

Temperatures lying under 15 °C (in our study, range 9–12 °C) should be assumed the suboptimal temperatures (for the period from egg fertilization up to finish of hatching) due to quite low level of accumulated mortality (value less than 50%). Nevertheless, we have no evidence about an accute boundary in term of lethality at low temperatures (boundary between still tolerated and lethal temperature) in range under 9 °C (generally, temperature 9 °C should be sublethal, in accordance to common situation in cyprinids – Wieser (1991)). However, it is highly recommended to distinguish and evaluate an impact of temperatures 9 and 12 °C (temperatures close to the lower boundary of the thermal tolerance range as Pepin *et al*. (1997) in Atlantic cod) on the embryonic and larval period up to finish of hatching only (survival over 50%), in contrast to possible other effect of these lower temperatures in context of the whole early ontogeny (it is a topic for the next possible study).

Generally, according to Wieser's (1991) classification, weatherfish (*M. fossilis*) as well as the related species, spined loach (*Cobitis taenia* sensu L.) (Bohlen, 2003), belongs to the warm-mesothermic species (thermal optimal range during early ontogeny 15–24 °C), but does not tolerate such a high temperature as a close-related species, oriental weatherfish (*M. anguillicaudatus*). In this Asian loach species, Watanabe *et al.* (1948) described common hatching of larvae at 28–30 °C, Kubota and Matsui (1955) considered the suitable temperatures for early ontogeny in range 20–28 °C (with optimum at 25 °C, or even at 25–27 °C according to Wang *et al.* (2008)).

Further studies (undertaking not only under laboratory conditions, but also in the wild) should be directed to understanding of interactions among early ontogeny, behaviour and exogenous factors influencing early-life history (especially in order to follow an exact role of early-life history characters determining survival), towards the management of the natural population of this hidden living fish species.

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# TEMPERATURE-INDUCED ONTOGENETIC PLASTICITY IN WEATHERFISH *Misgurnus fossilis* (Cypriniformes, Cobitidae) DURING EMBRYONIC AND LARVAL PERIODS

*Bořek Drozd, Martin Bláha, Jitka Hamáčková, Jan Kouřil*

*University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Research Institute of Fish Culture and Hydrobiology Vodňany, Zátiší 728/II, 389 25 Vodňany, Czech Republic*

## ABSTRACT

Weatherfish *M. fossilis* (L.) early life history (from egg activation to absolute yolk sac resorption) as evaluated at 9–36 °C (interval 3 °C) is overall within a viable temperature range for embryonic (9–24 °C; $t_0 = 8.36$ °C; $D°_{eff} = 28.7$) and larval (15–24 °C; $t_0 = 6.92$ °C; $D°_{eff} = 90.91$) periods. This is a truly thermal-independent process in terms of specimen outward appearances, as similar size and developmental stage [at $H_{50}$, Kostomarova's (1975) stage 37 at 9–18 °C and stage 36 at 21–24 °C] were achieved at each of the key events regardless of temperature, and of yolk sac utilization efficiency. There is by contrast, however, a time aspect in evaluating the onset of any crucial event (temporal prolongation was observed at lower temperatures) and in assessing any rate (survival, developmental and growth rate including also temperature $Q_{10}$ coefficients). Temperatures within the survival and developmental optima (15–24 °C and 15–21 °C for embryonic and larval period, respectively) accelerated development in terms of incubation, as well as hatching period duration and observation of the digestive system activation (Ac), onset of mixed (S) and exogenous (Re) feeding during different intervals of the embryonic and larval periods in a uniform (inversely proportional) way. An absolute lethal effect of higher temperatures (27–36 °C) manifested itself already during early cleavage [all eggs died in the stages of 4 blastomers (30–36 °C) or 8 blastomers (27 °C)], but in the case of lower temperatures (9 and 12 °C) this was not expressed until Re (resulting in large tolerance to low temperatures for relatively long periods as a response to periodic water temperature disturbances at the spawning localities). The two-stage curve (with an inflection region shortly after S) provided a useful model for evaluating growth and other morphometric characteristics. Regardless of temperature, larvae did not fully deplete the yolk sac until the gut had become a functional additional respiratory organ.

*Keywords:* development; growth; Misgurnus fossilis; survival; temperature requirement; yolk utilization

## 1.    INTRODUCTION

Loaches of the genus *Misgurnus* show a typical Holarctic distribution, but only one species – weatherfish *Misgurnus fossilis* (L.) – inhabits freshwaters throughout Europe (Kottelat and Freyhof, 2007). This nearly non-migrating species prefers slowly flowing or stagnant waters overgrown with water vegetation (Meyer and Hinrichs, 2000; Pekarik et al., 2008) from Northern France to Western Russia (but excluding Great Britain, Scandinavia and the Mediterranean) (Kottelat and Freyhof, 2007). Water quality degradation, along with drying or direct destruction of naturally occurring habitats during the second half of the 20th century led to dramatic decrease in its abundance across the whole of Europe and subsequently to its high species protection ratings (Kottelat and Freyhof, 2007). *M. fossilis* is today

included in many red lists of endangered fishes in Europe and under Annex II of Council Directive 92/43/EEC.

Fishes, such as barbel *Barbus barbus* (L.) or nase *Chondrostoma nasus* (L.) (Mann, 1996), comprise a very important indicator group for estimating river integrity (Karr, 1991). As it is true in rheophilic species, *M. fossilis* is among those fishes crucial for explaining river inundation areas' importance in respect to successful fish reproduction, as well as to distribution and dynamics of early developmental stages (Ward, 1998) that present the key events for fish recruitment and population dynamics (Kamler, 1992; Schiemer et al., 2003).

Fish ontogeny may be influenced by many and various biotic and abiotic factors, such as oxygen concentration (Kotlyarevskaja, 1967; Keckeis et al., 1996; Bohlen, 2003), pH (Prokes et al., 1998), salinity (Bohlen, 1999a) or water current (Schiemer et al., 2003). Temperature, however, is believed to be one of the most important such factors, impacting on sex determination (Conover and Kynard, 1981), development and growth (Penaz et al., 1983; Kamler et al., 1998), maximum swimming speed (Green and Fisher, 2004) and morphometric features plasticity (Stouracova et al., 1988; Penaz et al., 1989).

Consequently, an understanding of temperature's impact on a fish's early life history is a key to understanding fish egg and early developmental stages distribution and dynamics, as well as mechanisms for adapting (Klimogianni et al., 2004) to fluctuating environmental conditions within a river inundation area over the season (Ward, 1998; Pekarik et al., 2008).

To date, however, there is insufficient knowledge concerning the thermal limits of early life history in *M. fossilis* (Kostomarova, 1975; Alexeeva and Ozernyuk, 1987; Drozd et al., 2009). In order to predict wild population dynamics and life demands of *M. fossilis* for purposes of effective conservation management, the present study thus experimentally evaluates the sensitivity of embryonic and larval development in *M. fossilis* to temperature and its real thermal requirements during the period from egg activation to the onset of exogenous feeding.

## 2. MATERIALS AND METHODS

### 2.1. BROODSTOCK AND STRIPPING

A total 7 specimens of *M. fossilis* (4 females: $L_S$ = 223–241 mm, wet mass = 48–65 g; 3 males: $L_S$ = 167–190 mm, wet mass = 20–30 g) were captured in floodplain area pools on upper reaches of the Lužnice R. (tributary of the Vltava R., Elbe R. basin, North Sea drainage) close to Majdalena (48°97´ N; 14°86´ E) in South Bohemia, Czech Republic during April 2007. All specimens were transferred to the University of South Bohemia and held in aquaria (volume = 30 l, temperature = 16–18 °C).

The fish were induced on 23 April 2007 to spawn in response to gonadotrophin contained in carp pituitary intramuscularly injected at one dose of 5.00 mg.kg$^{-1}$ b.m. (body mass) for males and two doses of 0.50 and 4.50 mg.kg$^{-1}$ b.m., respectively (interval: 12 hours) for females. Hand-stripped oocytes were dry fertilized with pooled milt at 18 °C. Prior to any handling, adult fish were anaesthetized with 0.04 ml.l$^{-1}$ clove oil (Dr. Kulich Pharma, Ltd., Czech Republic). After stripping, all fish were released back to their original localities.

### 2.2. EGG AND LARVAE CULTURE SYSTEM

Fertilized and unstuck eggs (mean ± S.D., wet mass = 0.88 ± 0.08 mg, diameter = 1.42 ± 0.11 mm) and larvae were incubated at 10 temperatures (mean ± S.D., 9.00 ± 0.40, 12.00 ± 0.20, 15.00 ± 0.20, 18.00 ± 0.40, 21.00 ± 0.30, 24.00 ± 0.40, 27.00 ± 0.20, 30.00 ± 0.20, 33.00 ± 0.30, 36.00 ± 0.20 °C).

Temperatures were sustained using temperature-controlling refrigeration / heating system and measured using data loggers (RT–F53, Qi Analytical, Ltd., Czech Republic) every 0.5 h (range ca ± 0.5 °C). Temperature-acclimated reservoirs (volume = 2000 ml) with oxygen saturated water served as a source for culture water replacement in each temperature unit. Concentration of dissolved oxygen ($O_2$ saturation in range: 70–100%) and pH (range: 7.5–8.5) were measured twice daily using a handheld oxygen-pH meter (Oxi 315i, WTW GmbH, Germany) with additional regular monitoring of $NH_4^+$, $NO_3^-$, $NO_2^-$, and chemical oxygen demand at 3-day intervals. Photoperiod (light intensity of 50–100 lx at the water surface) was the same (12L:12D) at all temperatures.

Two types of incubators (type I: glass beaker, volume = 250 ml; type II: transparent plastic box, volume = 2000 ml) placed in bath units (glass aquaria, water volume = 25 l) and connected to the temperature-controlling system were used. Type I (in triplicates, N = 50 eggs.beaker$^{-1}$) was used for monitoring survival and all developmental events in time while type II (in duplicate, N = 1000 eggs. box$^{-1}$) was used for sampling at crucial developmental events.

After hatching (1–2 days prior to the onset of mixed feeding) larvae were fed to excess every 3 h with live brine shrimp *Artemia salina* (L.) nauplii and fine sieved pond zooplankton (up to 0.3 mm), which were the only food sources until the experiment was completed. The study continued for 63 days, which was 4 days after absolute yolk sac resorption was achieved at the lowest temperature where possible.

## 2.3.   DATA COLLECTION AND ANALYSIS

The approach of Kamler (2002) was used to classify early fish life history and discriminate key ontogenetic events (designated as crucial events or thresholds), i.e. egg activation (Fe), hatching (H), first exogenous food intake (S; observation of the first *A. salina* nauplii in the gut) and absolute yolk sac depletion (Re; full resorption of the last energy reserves stored in the yolk sac and the onset of exogenous feeding too). Within the period after hatching one additional crucial event related to digestion was discerned, i.e. digestive system activation (Ac; appearance in the gut of ovoid clusters white-yellow in colour indicating a bile secretion and activation of digestive enzymes) (Penaz, 2001). Hatching was considered to be the true onset of the larval period. Developmental stages were determined using criteria published by Kryzanovskij (1949) and Kostomarova (1975) in *M. fossilis* and/or by Fujimoto et al. (2006) in oriental weatherfish *Misgurnus anguillicaudatus* (Cantor). The time scale used for the development is presented as days post-egg activation (dPF).

Fish samples (usually 5–10, at the crucial events at least 30 eggs and larvae, respectively) were taken at regular 0.5 h intervals from the egg activation [0 hour post-egg activation (0 dPF)] to 1 day post-egg activation (1 dPF), 2 h (period: 2–3 dPF), 6 h (period: 4–8 dPF) and 12 h (period: 9–63 dPF), respectively. The incubated eggs and larvae designated for next morphometric examination (at least 500 individuals per temperature, when possible) were preserved in 4% phosphate buffered paraformaldehyde solution for 10–50 days [in accordance with a recommendation of Lusk and Pokorny (1964) for optimal fish material conservation and examination].

At each temperature (incubator type I), mortality of eggs and/or larvae was recorded in intervals of 0.5–6 h (9–24 °C) and 0.5 h (27–36 °C). White opaque eggs and/or larvae were identified as dead individuals and were siphoned off. The accumulated survival rate was calculated from the difference between the initial numbers of eggs after activation and numbers of survivors at each crucial event (assessed at Fe, $H_{50}$, S, Re, 4 (3) days after Re), when possible (investigations at temperatures 27–36 °C ended earlier because all eggs had died before the start of hatching).

Incubation period (τ, days) was taken as the time interval between Fe and H (Kamler, 2002) within

which four crucial events were distinguished: (1) start of hatching (point of hatching of 5% of individuals), (2) $H_{50}$ (point of hatching of 50% of individuals), (3) $H_{75}$ (point of hatching of 75% of individuals) and (4) finish of hatching (point of hatching of 95% of individuals). Hatching period duration was designated as the time interval from start to finish of hatching (Kamler, 2002).

For evaluating the relationship between developmental rate ($V = \tau^{-1}$; days$^{-1}$) and incubation temperature ($t$; °C) during the embryonic ($V_H$; from Fe to $H_{50}$) and larval ($V_{post-H}$; from $H_{50}$ to Re) periods, the following linear model was used:

$$V = a + b \cdot t$$ (Kamler, 2002)

from which the next two biologically meaningful parameters can be derived. Temperature of biological zero (threshold temperature at which ontogeny is theoretically stopped, t0):

$$t_0 = -a / b$$ (Kamler, 2002),

and the effective day-degrees (number of day-degrees above the temperature of biological zero, $D°_{eff}$):

$$D°_{eff} = \tau \cdot (t - t_0) = b^{-1}$$ (Kamler, 2002).

The lowest viable temperature ($t_{lowest}$) was computed from directly observed mortality data using the following theoretical mathematical formula:

$$t_{lowest} = 1.34 + 0.929 \cdot t_0$$ (Kamler, 2002).

Sigmoid time course for the cumulative percentage of hatched individuals (P) against time [as demonstrated for example in *C. nasus* by Kamler et al. (1998)] was linearized by converting percentages to logits:

$$logit = \log_{10} [0.01 \cdot P / (1 - 0.01 \cdot P)]$$ (Berkson, 1944)

and plotting against the logarithm of time. The predicted times for start of hatching, $H_{50}$, $H_{75}$ and finish of hatching were computed from these linear regressions (Kamler et al., 1998; Kamiński et al., 2006).

The acceleration in rates (V) by temperature ($t$; °C) was expressed by the temperature coefficient:

$$Q_{10} = (V_2 / V_1)^{10 / (t^2 - t^1)}$$ (Kamler, 2002),

where the coefficients for developmental rate ($Q_{10\,dev}$) and growth rate ($Q_{10\,gr}$) were taken from: $V_{dev} = \tau^{-1}$ (in days$^{-1}$) or $V_{gr}$ = SGR [specific growth rate in %.day$^{-1}$; SGR = 100 . (ln $L_{T2}$ - $L_{T1}$) . ($t_2 - t_1$), where t is time in days], respectively.

In order to examine fixed individuals taken randomly at each sample point, a binocular microscope (Olympus SZ 40, SZX 9) fitted with a phototube and digital camera (Sony Progressive 3CCD and Olympus Camedia C5060UW) was used for taking pictures. Digital images were then processed by video image analysis software (MicroImage version 3.0.1 for Windows) to analyse selected morphometric characteristics (Table 1). Yolk sac volume (YsV; in μl) was computed according to the formula for the prolate spheroid:

$YsV = [\pi / 6] \cdot YsL \cdot YsD^2$                                   (Blaxter and Hempel, 1963),

where YsL and YsD represent yolk sac length and yolk sac depth (Table 1), respectively.

**Table 1.**  *M. fossilis. Morphometric characteristics measured in present study.*

| Character | Abbreviation | Description |
|---|---|---|
| Total length | $L_T$ | From tip of snout to posterior margin of caudal part of fin fold (later caudal fin) |
| Standard length | $L_S$ | From tip of snout to posterior tip of the caudal peduncle |
| Pre-anal length | prAn | From tip of snout to anus |
| Post-anal length | psAn | From anus to posterior tip of the caudal peduncle |
| Pre-orbital length | prOrb | From tip of snout to anterior margin of eye |
| Orbital length | Orb | From anterior to posterior margin of eye |
| Yolk sac length | YsL | Horizontal maximal size of yolk sac |
| Yolk sac depth | YsD | Vertical maximal size of yolk sac |

One-way ANOVA (including Tukey HSD test in a next step) was used to evaluate possible significant difference in the accumulated survival rate, incubation period, digestion, total length of newly hatched larvae, and yolk sac volume (all of which were evaluated at the crucial events mentioned above) within the temperatures (no significant differences were found among triplicates of each temperature, $P = 0.05$) and among constituent temperature groups. Data for the amplitude of the incubation period, digestion (both evaluated at crucial events mentioned above), hatching in logits, developmental rate (for periods Fe–$H_{50}$ and $H_{50}$–Re), morphometric characteristics ($L_T$, $L_S$, prAn, psAn, prOrb, Orb) and depletion of yolk sac volume (YsV) were analysed by multiple regression to determine and visualize graphically (2D graphs were used) the relationship between each magnitude and temperature. Linear or non-linear regressions fitted by least squares iteration through all raw data to logarithmic, polynomial or sigmoidal dose-response functions were used for these purposes. The programmes Statistica 7.0 (StatSoft, Inc.) and Table Curve 2D (AISN Software, Inc.) were used for data analyses. Data from water temperatures 27, 30, 33 and 36 °C were excluded from further statistical analyses due to the lethal impact on egg survival already during the early embryonic period.

## 3.    RESULTS

### 3.1.  DEVELOPMENT

#### 3.1.1. Incubation period

The number of days from Fe to: (1) start of hatching (ranged from 12.87 to 1.61 days on average), (2) $H_{50}$ (ranged from 17.62 to 1.77 days on average), (3) $H_{75}$ (ranged from 18.13 to 1.85 days on average) and (4) finish of hatching (ranged from 18.61 to 1.99 days on average) all decreased with rising temperature (Table 2).

Regressions of both incubation period (evaluated at the four crucial events mentioned above, plotted on a log time axis) (Figure 1) and percentages of hatched individuals (converted to logits and plotted against the logarithm of time) (Figure 2) against temperature were highly significant (Table 3).

**Table 2.** *Effect of incubation temperature on survival rate (from egg activation up to 3–4 days after absolute yolk sac depletion; in %), incubation period duration (in dPF or days), total length (in mm), yolk sac volume (in mm³) and digestion (in dPF) evaluated at the key ontogenetic events (data shown as mean ± S.D.) in M. fossilis. Data measured at temperatures 27–36 °C are unified into one column (or not shown) due to lethal impact of these temperatures already during the embryonic period. The predicted times of start of hatching, $H_{50}$, $H_{75}$ and finish of hatching are computed from the linear regressions of cumulative percentage of hatched individuals (in logits) and logarithm of time in relation to temperature (see Table III).*
*Start of hatching, point of hatching of 5% of individuals; $H_{50}$, point of hatching of 50% of individuals; $H_{75}$, point of hatching of 75% of individuals; Finish of hatching, point of hatching of 95% of individuals; Total hatching period duration, time interval between start and finish of hatching; Digestive system activation, appearance in the gut of void clusters white-yellow in colour representing digestive enzymes activation; First exogenous food intake, observation of the first ingested Artemia nauplii in the gut; Absolute yolk sac depletion, full resorption of the last energy reserves stored in the yolk sac.*

| Temperature (°C) | | 9 | 12 | 15 | 18 | 21 | 24 | 27–36 |
|---|---|---|---|---|---|---|---|---|
| **Survival (%)** | | | | | | | | |
| Egg activation | | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 | 100.00 ± 0.00 |
| $H_{50}$ | | 52.50 ± 22.44 | 58.56 ± 21.91 | 68.16 ± 9.76 | 71.00 ± 10.91 | 73.18 ± 9.38 | 66.99 ± 10.27 | 0.00 ± 0.00 |
| First exogenous food intake | | 24.95 ± 14.10 | 58.23 ± 20.77 | 64.87 ± 11.50 | 67.20 ± 9.01 | 70.76 ± 6.72 | 60.54 ± 11.96 | 0.00 ± 0.00 |
| Absolute yolk sac depletion | | 17.71 ± 15.26 | 32.15 ± 8.92 | 63.57 ± 10.70 | 67.20 ± 10.01 | 69.18 ± 7.32 | 57.82 ± 9.64 | 0.00 ± 0.00 |
| 3–4 days after absolute yolk sac depletion | | 0.00 ± 0.00 | 0.00 ± 0.00 | 62.91 ± 11.00 | 66.53 ± 10.10 | 65.67 ± 2.37 | 41.50 ± 14.00 | 0.00 ± 0.00 |
| **Incubation period (dPF [1] or days [2])** | | | | | | | | |
| Start of hatching[1] | observed | 12.87 ± 0.29 | 7.11 ± 0.26 | 4.60 ± 0.16 | 2.97 ± 0.10 | 1.95 ± 0.11 | 1.61 ± 0.04 | – |
| | predicted | 13.33 | 7.32 | 4.83 | 3.04 | 1.88 | 1.50 | – |
| $H_{50}$[1] | observed | 17.62 ± 0.33 | 7.99 ± 0.23 | 5.38 ± 0.16 | 3.23 ± 0.10 | 2.19 ± 0.04 | 1.77 ± 0.03 | – |
| | predicted | 15.98 | 8.17 | 5.30 | 3.29 | 2.16 | 1.70 | – |
| $H_{75}$[1] | observed | 18.13 ± 0.27 | 8.28 ± 0.10 | 5.54 ± 0.12 | 3.38 ± 0.06 | 2.24 ± 0.04 | 1.85 ± 0.03 | – |
| | predicted | 17.10 | 8.52 | 5.49 | 3.38 | 2.27 | 1.83 | – |
| Finish of hatching[1] | observed | 18.61 ± 0.07 | 8.57 ± 0.27 | 5.83 ± 0.10 | 3.63 ± 0.10 | 2.40 ± 0.07 | 1.99 ± 0.03 | – |
| | predicted | 19.16 | 9.12 | 5.82 | 3.55 | 2.48 | 2.07 | – |
| Total hatching period duration[2] | observed | 5.46 ± 0.18 | 1.67 ± 0.19 | 1.23 ± 0.20 | 0.66 ± 0.12 | 0.45 ± 0.06 | 0.38 ± 0.05 | – |
| | predicted | 5.83 | 1.80 | 0.98 | 0.51 | 0.59 | 0.57 | – |
| **Total length (mm)** | | | | | | | | |
| $H_{50}$ | | 4.31 ± 0.15 | 4.67 ± 0.24 | 4.29 ± 0.23 | 4.29 ± 0.24 | 4.22 ± 0.24 | 4.30 ± 0.18 | – |
| First exogenous food intake | | 5.95 ± 0.49 | 6.52 ± 0.31 | 6.64 ± 0.32 | 6.64 ± 0.21 | 6.54 ± 0.31 | 6.47 ± 0.38 | – |
| Absolute yolk sac depletion | | 6.03 ± 0.25 | 7.21 ± 0.18 | 7.40 ± 0.34 | 7.24 ± 0.54 | 7.12 ± 0.09 | 7.23 ± 0.15 | – |

***Table 2.*** *continuation*

| | Yolk sac volume [mm³, (% of the yolk sac volume at $H_{50}$)] | | | | | | |
|---|---|---|---|---|---|---|---|
| $H_{50}$ | 1.48 ± 0.26 (100%) | 1.81 ± 0.18 (100%) | 1.49 ± 0.18 (100%) | 1.50 ± 0.21 (100%) | 1.45 ± 0.16 (100%) | 1.51 ± 0.14 (100%) | – |
| First exogenous food intake | 0.06 ± 0.05 (4.05%) | 0.12 ± 0.05 (6.55%) | 0.10 ± 0.04 (6.65%) | 0.07 ± 0.04 (4.65%) | 0.05 ± 0.03 (3.65%) | 0.06 ± 0.03 (3.85%) | – |
| **Digestion (dPF)** | | | | | | | |
| Digestive system activation | 59.00 | 19.50 | 13.50 | 8.50 | 6.00 | 5.50 | – |
| First exogenous food intake | 57.00 | 19.50 | 13.50 | 9.00 | 6.50 | 6.00 | – |
| Absolute yolk sac depletion | 59.00 | 27.00 | 17.50 | 11.00 | 8.00 | 7.50 | – |
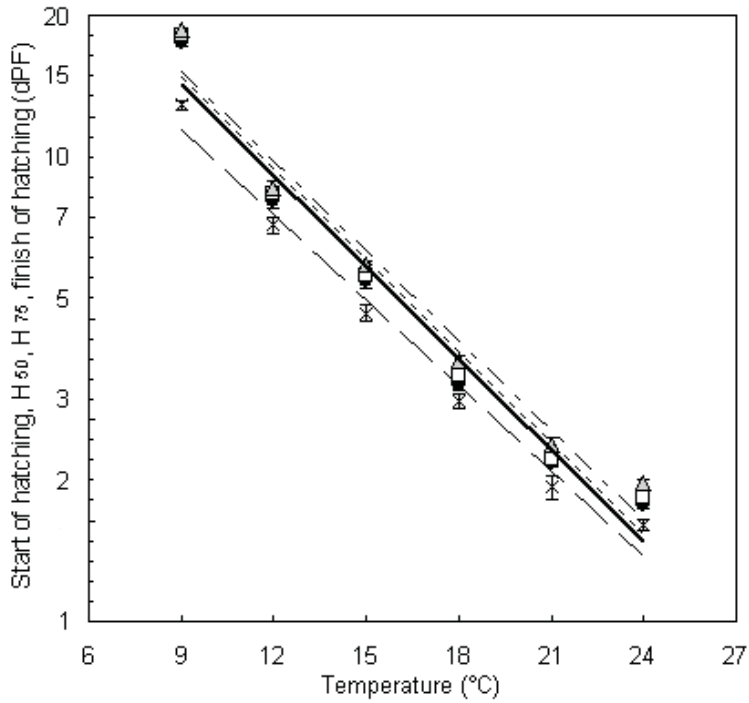
**Figure 1.** *Effect of temperature on incubation period duration (shown as mean ± S.D.) evaluated at four crucial moments, i.e. at the start of hatching (point of hatching of 5% of individuals), $H_{50}$ (point of hatching of 50% of individuals), $H_{75}$ (point of hatching of 75% of individuals) and finish of hatching (point of hatching of 95% of individuals) in M. fossilis.*
*Data for start of hatching, $H_{50}$, $H_{75}$ and finish of hatching are plotted on a logarithmic axis to achieve linearity (regression and statistical parameters are shown in Table 3).*

----×----, start of hatching; — • —, $H_{50}$; ····□····, $H_{75}$; ·--·△·--·, finish of hatching
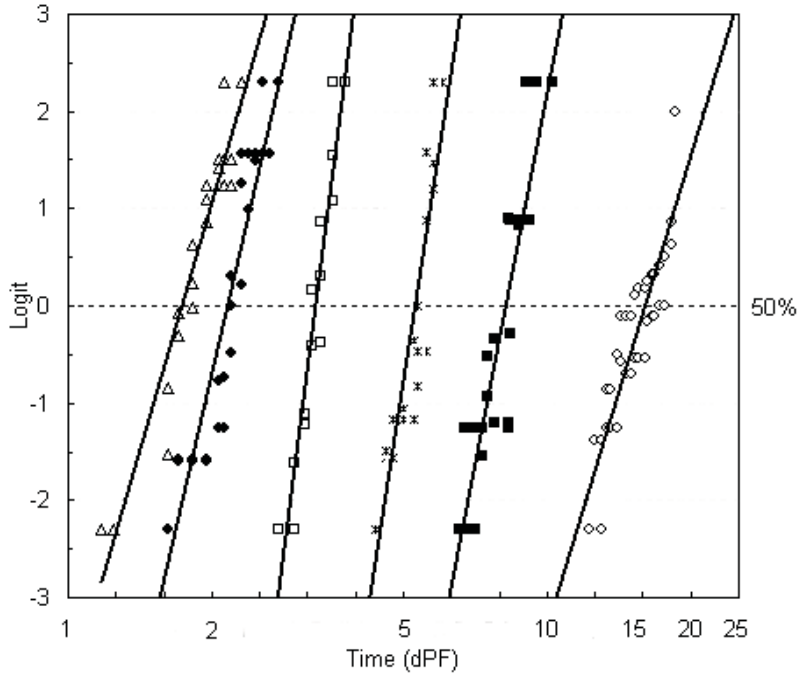
**Figure 2.** *Cumulative percentage of hatched individuals (converted to logits and plotted against the logarithm of the time) in relation to the incubation temperature in M. fossilis (regression parameters are shown in Table 3). $H_{50}$ (point of hatching of 50% of individuals) corresponds to logit = 0.*

○, 9 °C; ∎, 12 °C; ✳, 15 °C; □, 18 °C; •, 21 °C; △, 24 °C

**Table 3.** *Incubation period duration, developmental rate, digestion and cumulative percentage of hatched individuals (converted to logits and plotted against the logarithm of the time) in relation to incubation temperature (fitted by linear function $y = a + b . x$) in M. fossilis.*

*Start of hatching, point of hatching of 5% of individuals; $H_{50}$, point of hatching of 50% of individuals; $H_{75}$, point of hatching of 75% of individuals; Finish of hatching, point of hatching of 95% of individuals; Digestive system activation, appearance in the gut of ovoid clusters white-yellow in colour representing a bile secretion and activation of digestive enzymes; First exogenous food intake, observation of the first ingested Artemia nauplii in the gut; Absolute yolk sac depletion, full resorption of the last energy reserves stored in the yolk sac;*

*$V_H$, embryonic developmental rate; $V^{post-H}$, larval (post-hatching) developmental rate, i.e. V for period between $H_{50}$ and absolute yolk sac depletion (V in days$^{-1}$); F, test criterion; N, number of observations; D.f., degrees of freedom; $r^2$, correlation coefficient.*

| Event | Incubation period | | | | | | |
|---|---|---|---|---|---|---|---|
| | F | N | D.f. | P | $r^2$ | a | b |
| Start of hatching | 1339.73 | 30 | 1 | < 0.01 | 0.98 | 3.6923 | -0.1402 |
| $H_{50}$ | 831.72 | 30 | 1 | < 0.01 | 0.97 | 4.0206 | -0.1511 |
| $H_{75}$ | 844.77 | 30 | 1 | < 0.01 | 0.97 | 4.0486 | -0.1508 |
| Finish of hatching | 840.73 | 30 | 1 | < 0.01 | 0.97 | 4.0472 | -0.1474 |
| | Developmental rate | | | | | | |
| | F | N | D.f. | P | $r^2$ | a | b |
| $V_H$ | 167.51 | 6 | 1 | < 0.01 | 0.98 | -0.2909 | 0.0348 |
| $V_{post-H}$ | 133.91 | 6 | 1 | < 0.01 | 0.97 | -0.0761 | 0.0110 |
| | Digestion | | | | | | |
| | F | N | D.f. | P | $r^2$ | a | b |
| Digestive system activation | 38.27 | 6 | 1 | < 0.01 | 0.91 | 5.0406 | -0.1511 |
| First exogenous food intake | 34.87 | 6 | 1 | < 0.01 | 0.92 | 4.9300 | -0.1425 |
| Absolute yolk sac depletion | 58.03 | 6 | 1 | < 0.01 | 0.94 | 5.0550 | -0.1374 |
| Temperature (°C) | Hatching (in logits) | | | | | | |
| | F | N | D.f. | P | $r^2$ | a | b |
| 9 | 123.61 | 136 | 1 | < 0.01 | 0.81 | -19.5193 | 16.2185 |
| 12 | 102.73 | 125 | 1 | < 0.01 | 0.89 | -24.4213 | 26.7670 |
| 15 | 193.23 | 121 | 1 | < 0.01 | 0.91 | -23.0457 | 31.8127 |
| 18 | 150.16 | 115 | 1 | < 0.01 | 0.96 | -19.7371 | 38.1801 |
| 21 | 163.28 | 126 | 1 | < 0.01 | 0.93 | -7.0996 | 21.2752 |
| 24 | 179.69 | 122 | 1 | < 0.01 | 0.95 | -3.4757 | 15.0780 |

The predicted times for the start of hatching, $H_{50}$, $H_{75}$ and finish of hatching computed from linear regressions for hatching in logits (Table 3) lay within the mean ± S.D. intervals for observed values (Table 2) at all temperature treatments except for temperatures 9 °C (at all crucial events mentioned above) and 12 °C (at $H_{75}$ and finish of hatching), which were most probably affected by poorer correlation of hatching and time ($r^2 = 0.81$ and 0.89, respectively) at these temperatures (Table 3).

Hatching was more synchronous at higher temperatures, indicating that total hatching period duration (ranging from 5.46 to 0.38 days on average) decreased with rising temperature (Table 2).

### 3.1.2. Digestion

Larvae incubated at temperatures 9, 12, 15, 18, 21 and 24 °C activated their digestive systems (Ac) at the ages of ca 59, 19.5, 13.5, 8.5, 6 and 5.5 dPF, respectively; ingested the first exogenous food (S) at the ages of ca 57, 19.5, 13.5, 9, 6.5 and 6 dPF (yolk sac volume reached ca 4, 6.5, 6.5, 4.5, 4 and 4% of the yolk sac volume at $H_{50}$, respectively); and switched to exclusively exogenous nutrition (i.e. yolk sac was fully depleted, Re) at the ages of ca 59, 27, 17.5, 11, 8 and 7.5 dPF (Table 2).

At temperatures 18, 21 and 24 °C digestive system activation preceded the first exogenous food intake by ca 0.5 day, which was followed by the onset of exogenous feeding ca 1.5–2 days later. Nevertheless, at both temperatures 12 and 15 °C the digestive system activation and first exogenous food intake proceeded almost simultaneously (it was impossible to separate these two crucial events in time) and were followed by the onset of exogenous feeding 7.5 or 4 days thereafter, respectively. At the lowest incubation temperature of 9 °C, however, larvae started to ingest the first exogenous food ca 2 days prior to both digestive system activation and the onset of exogenous feeding (i.e. absolute yolk sac depletion) that had occurred simultaneously.

Regressions of digestive system activation, first exogenous food intake, absolute yolk sac depletion (plotted on a log time axis) and temperature were all highly significant (Table 3) and suggested an inversely proportional relationship between digestion and temperature (Figure 3).
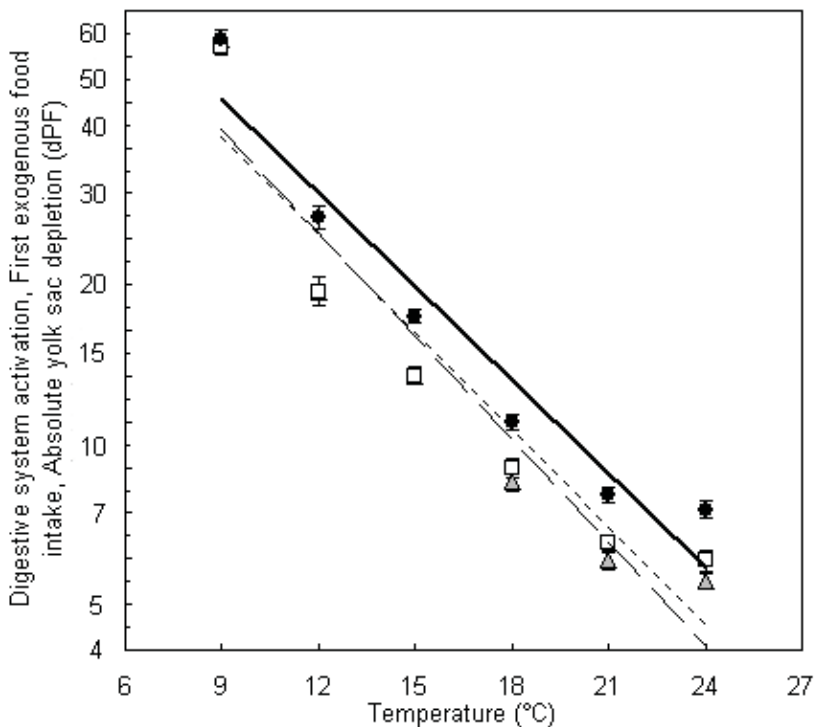


**Figure 3.** *Effect of temperature on digestive system activation* (---△---), *first exogenous food intake* (····□····) *and absolute yolk sac depletion* (— ● —) *(shown as mean ± S.D.) in M. fossilis (regression and statistical parameters are shown in Table 3).*
*Data for all investigated parameters are plotted on logarithmic axis to achieve linearity.*

### 3.1.3. Developmental rate

The developmental rates for both embryonic ($V_H$) and larval ($V_{post-H}$) periods increased with rising temperature and decreasing age, with steady state or plateau at 21 and 24 °C during the post-hatching interval at all crucial events (Ac, S, Re) (Table 4). From a highly significant linear relationship (Table 3) between developmental rate over the embryonic (i.e. from Fe to $H_{50}$) and larval (i.e. from $H_{50}$ to Re) periods and temperature (Figure 4) it was found that in *M. fossilis* embryonic development is theoretically arrested at 8.36 °C; hatching ($H_{50}$) occurs at $D°_{eff}$ = 28.74 (inter-specific comparison of both is shown in Figure 5); larval development is theoretically arrested at 6.92 °C; and the yolk sac is full resorbed (Re) after the next 90.91 effective day-degrees. The lowest viable temperatures were computed at 9.11 and 7.77 °C in terms of embryonic and larval survival, respectively. (Complete mortality 3–4 days post-Re was observed, however, in larvae incubated at 9 and 12 °C. See Discussion for more on developmental vs. physiological requirements of *M. fossilis*).

**Table 4.** *Accelerations of developmental and growth rates by temperature ($Q_{10\,dev}$, $Q_{10\,gr}$) from egg activation to hatching ($H_{50}$) and from hatching to absolute yolk sac depletion (evaluated for intervals from $H_{50}$ up to digestive system activation, first exogenous food intake, and absolute yolk sac depletion) in M. fossilis. Data in parentheses represent ranges of two adjacent temperatures.*

*$H_{50}$, point of hatching of 50% of individuals; V, developmental rate (in day$^{-1}$); $Q_{10}$ dev, temperature coefficient for developmental rate; $Q_{10\,gr}$, temperature coefficient for growth rate; SGR, specific growth rate (in %.day$^{-1}$)*

| Temperature (°C) | Developmental acceleration rate | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Egg activation – $H_{50}$ | | $H_{50}$ – Digestive system activation | | $H_{50}$ – First exogenous food intake | | $H_{50}$ – Absolute yolk sac depletion | |
| | V (day$^{-1}$) | $Q_{10\,dev}$ | V (day$^{-1}$) | $Q_{10\,dev}$ | V (day$^{-1}$) | $Q_{10\,dev}$ | V (day$^{-1}$) | $Q_{10\,dev}$ |
| 9 | 0.06 | | 0.02 | | 0.03 | | 0.02 | |
| 12 (9–12) | 0.13 | 13.59 | 0.09 | 72.08 | 0.09 | 61.14 | 0.05 | 13.52 |
| 15 (12–15) | 0.18 | 3.49 | 0.13 | 3.35 | 0.13 | 3.35 | 0.08 | 4.63 |
| 18 (15–18) | 0.33 | 7.54 | 0.18 | 3.49 | 0.17 | 2.61 | 0.13 | 3.86 |
| 21 (18–21) | 0.50 | 3.86 | 0.25 | 2.89 | 0.22 | 2.61 | 0.17 | 2.61 |
| 24 (21–24) | 0.67 | 2.61 | 0.25 | 1.00 | 0.22 | 1.00 | 0.17 | 1.00 |

| Temperature (°C) | Growth acceleration rate | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | $H_{50}$ – Digestive system activation | | $H_{50}$ – First exogenous food intake | | $H_{50}$ – Absolute yolk sac depletion | |
| | SGR (%.day$^{-1}$) | $Q_{10\,gr}$ | SGR (%.day$^{-1}$) | $Q_{10\,gr}$ | SGR (%.day$^{-1}$) | $Q_{10\,gr}$ |
| 9 | 0.78 | | 0.82 | | 0.81 | |
| 12 (9–12) | 2.91 | 78.74 | 2.91 | 69.20 | 2.29 | 31.96 |
| 15 (12–15) | 5.47 | 8.20 | 5.46 | 8.12 | 4.54 | 9.81 |
| 18 (15–18) | 7.18 | 2.48 | 7.27 | 2.60 | 6.54 | 3.36 |
| 21 (18–21) | 10.11 | 3.13 | 9.71 | 2.62 | 8.69 | 2.58 |
| 24 (21–24) | 8.99 | 0.68 | 9.09 | 0.80 | 8.66 | 0.99 |

The developmental stages of newly hatched *M. fossilis* larvae tended slightly to increase with declining temperature. Eggs incubated at 9, 12, 15 and 18 °C hatched at stage 37 (first visible germ of pectoral fin, head noticeably separated from yolk sac, 17 caudal somites, straight caudal part; classification by Kostomarova, 1975), i.e. at the 50-somite stage [approach of Fujimoto et al. (2006) in *M. anguillicaudatus*]. Those kept at 21 and 24 °C hatched at stage 36 (first visible otoliths in otic capsule, head slightly separated from the yolk sac, 10–13 caudal somites, still curved caudal part), i.e. at the 44-somite stage. Regardless of temperature, all *M. fossilis* larvae after the onset of exogenous feeding (Re) showed traits associated with two of Kryzanovskij's (1949) late larval stages, i.e. stages at the ages of 12 and 26 dPF (formed two pairs of barbs, outer filamentous gills almost resorbed, gill membrane incompletely covering gill arches, segmental blood vessels formed in dorsal and anal part of fin-fold, no yolk sac rudiments).
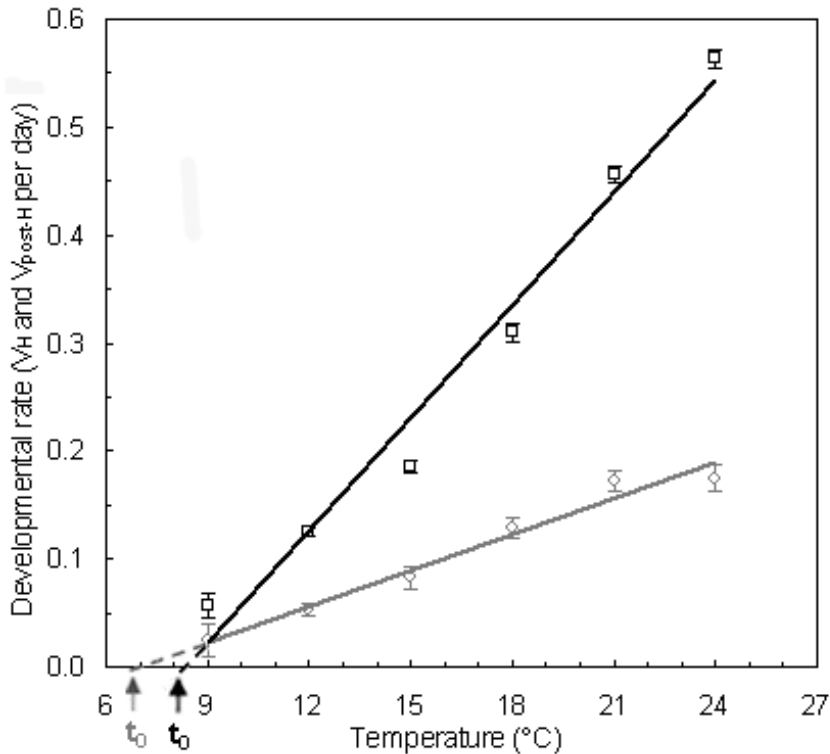


**Figure 4.** *Linear model of relationship between developmental rate ($V_H$ and $V_{post-H}$) and temperature ($V = a + b \cdot t$) in M. fossilis. $V_H$, embryonic developmental rate ($\square$, black line and $t_0$); $V_{post-H}$, larval (post-hatching) developmental rate, i.e. V for period between $H_{50}$ and absolute yolk sac depletion ($\bigcirc$, grey line and $t_0$). $V = \tau^{-1}$, where $\tau^{-1}$ represents hatching time (using mean hatching time $H_{50}$, i.e. the point of hatching of 50% of individuals; dPF) or time of absolute yolk sac depletion, respectively (regression parameters are shown in Table 3). ——, regression line $V_{H50}$; ——, regression line $V_{post-H}$; ▬ ▬ ▬, extrapolation of regression line for each period; t0, temperature of biological zero (threshold temperature at which ontogeny is theoretically arrested) for each period.*
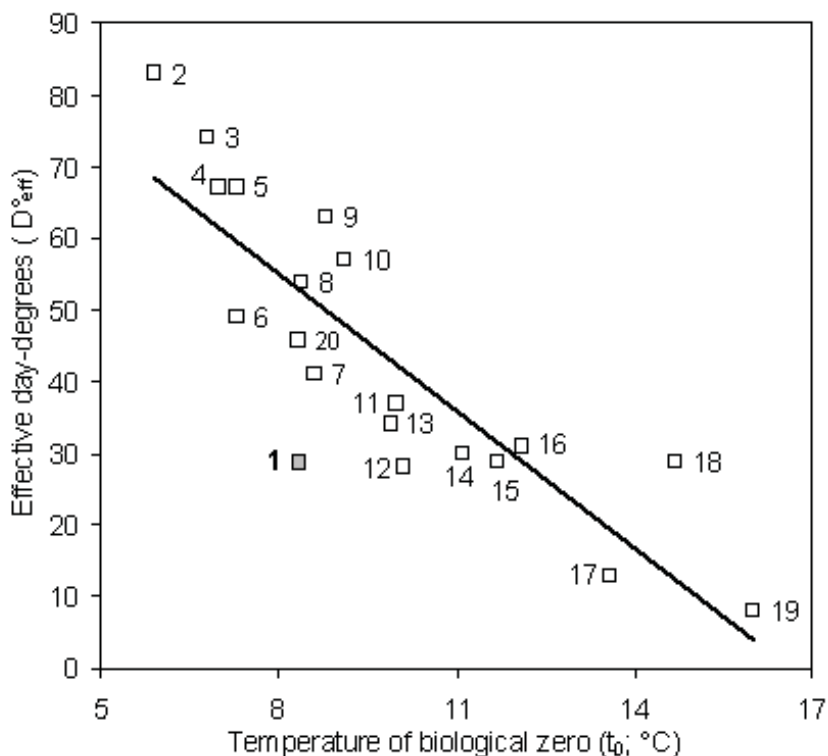
**Figure 5.** *Relationship between effective day-degrees (D°$_{eff}$) and temperature of biological zero (t$_0$) for embryonic development (i.e. from egg activation to H$_{50}$, i.e. to the point of hatching of 50% individuals) of M. fossilis (1, present study) and selected cyprinid species (2–19, literature data presented by Kamler, 2002; 20, Kamiński et al., 2006).*

*1, Misgurnus fossilis (L.); 2, Leuciscus idus (L.); 3, Aspius aspius (L.); 4, Gobio albipinnatus (Lukasch); 5, Rutilus rutilus (L.); 6–7, Abramis brama (L.); 8–9, Chondrostoma nasus (L.); 10, Scardinius erythrophthalmus (L.); 11, Alburnus chalcoides (Güldenstädt); 12, Vimba vimba (L.); 13–14, Cyprinus carpio L.; 15, Tinca tinca (L.); 16, Barbus barbus (L.); 17, Ctenopharyngodon idella (Valenciennes); 18, Carassius carassius (L.); 19, Hypophthalmichthys molitrix (Valenciennes); 20, Eupallasella percnurus (Pallas).*

## 3.1.4. Yolk utilization

Duration of yolk depletion (expressed as yolk sac volume decrease in time) was inversely proportional to incubation temperature during all distinguished phases, i.e. lag, decline, and terminal phases (Figure 6). Data were fitted significantly to the sigmoidal regressions (Table 5).

Yolk sac volume (Table 2) significantly differed by temperature at both crucial events, i.e. at mean hatching time (H$_{50}$) (F$_{5,114}$ = 36.73, P < 0.01) and first exogenous food intake (S) (F$_{5,114}$ = 42.04, P < 0.01). Tukey HSD test revealed (P = 0.05) that yolk amounts at H$_{50}$ differed significantly only for 12 °C and at S were significantly different for 9, 21, 24 °C from 12 and 15 °C, for 12 °C from 9, 18, 21 and 24 °C, and for 18 °C from 12 °C (Figure 7). The former, as well as the larger larvae (i.e. YsV and L$_T$ at H$_{50}$ significantly higher at 12 °C compared to other temperatures; see Figure 7) imply that both are probably induced by error of the collector, who selectively chose larger larvae (a rise of ca 0.30 mm

and 0.30 mm³ on average, respectively, at 12 °C compared to others) rather than by a real impact of low temperature (Kamler, 1992, 2002; Kamiński et al., 2006). On these grounds, data for the yolk sac volume, as well as for all morphometric characteristics, obtained at 12 °C and $H_{50}$ were excluded from the entire process of statistical evaluation.

Yolk utilization followed a three-phase sigmoidal pattern (Figure 6). The initial lag phase, during which there was ca 10% yolk depletion [compared to the original yolk reserves at hatching (denotes as Y.r.) expressed as the yolk sac volume at $H_{50}$], lasted ca 6.8, 2.5, 1.7, 1.0, 0.8 and 0.7 days at 9, 12, 15, 18, 21 and 24 °C, respectively. The decline (second) phase occurred as the yolk mass was rapidly depleted from ca 90% to ca 10% of Y.r. over a period taking ca 25.0 (32.6 dPF), 9.5 (14.1 dPF), 6.1 (10.5 dPF), 4.1 (6.0 dPF), 3.1 (4.4 dPF), 3.1 (4.0 dPF) days at 9, 12, 15, 18, 21 and 24 °C, respectively (numbers in brackets show a time of ca 50% of Y.r.). During the final, terminal phase, the remaining ca 10% of the yolk mass was slowly utilized over a period that lasted ca 10.0, 7.0, 4.3, 3.5, 2.0 and 2.0 days at 9, 12, 15, 18, 21 and 24 °C, respectively.

### 3.1.5. Growth and morphometry

Growth [expressed as increase of total length ($L_T$) in time] was inversely proportional to incubation temperature and usually followed a two-stage curve pattern (Figure 8). Data for $L_T$ were fitted significantly to polynomial regressions (Table 5). The $L_T$ curve inflection region at each temperature, which is followed by an interval of rapid increase in size, corresponded to the onset of mixed feeding. An exception to this rule is seen in the fitting of $L_T$ data from larvae reared at 9 °C to logarithmic regression, because of considerably slower growth compared to the other temperature groups (Figure 8).

Total length (Table 2) significantly differed by temperature at all key events, i.e. at mean hatching time ($H_{50}$) ($F_{5,114} = 6.24$, P < 0.01), first exogenous food intake (S) ($F_{5,114} = 31.87$, P < 0.01), and absolute yolk sac depletion (Re) ($F_{5,114} = 56.42$, P < 0.01). Tukey HSD test (P = 0.05) revealed that $L_T$ at $H_{50}$ differed significantly only at 12 °C (as noted above, however, data measured at 12 °C were omitted ex post from the entire process of statistical evaluation), but at S, as well as at Re, total length differed significantly only at 9 °C (shown as significantly smaller larvae) (Figure 7).

During the larval period, the specific growth rate in terms of total length (SGR, %.day⁻¹) ranged by interval as follows: (1) H–Ac from 0.78 to 10.11, (2) H–S from 0.82 to 9.71, and (3) H–Re from 0.81 to 8.79. That is to say, growth increased with rising temperature (up to 21 °C) in a directly proportional way and then moderately declined (Table 4).

Standard ($L_S$), pre-anal (prAn), post-anal (psAn), pre-orbital (prOrb) and orbital (Orb) lengths followed a similar pattern of development by temperature as did $L_T$. Data for $L_S$, prAn, psAn, prOrb and Orb were fitted significantly (Table 5) to polynomial (at 12–24 °C) and logarithmic (at 9 °C) regressions. A distinct inflection in the $L_S$, prAn and prOrb course (Figures 9, 10, 11) that is followed by an interval of rapid increase in size is connected with the first exogenous food ingestion (S), as in $L_T$. In the psAn curve, however, only an indistinct inflection point was observed (Figure 12), and in the Orb course a notable inflection point occurred even relatively long before the onset of mixed feeding itself (Figure 13).

### 3.1.6. Increase in rates

Both developmental rate and growth rate (evaluated using temperature coefficients $Q_{10\,dev}$ and $Q_{10\,gr}$, respectively) for Fe–$H_{50}$, $H_{50}$–Ac, $H_{50}$–S and $H_{50}$–Re intervals, were strongly enhanced by temperature in an inversely proportional manner, i.e. they declined with rising temperature (Table 4). This general

pattern was violated for the embryonic period (Fe–H$_{50}$) within the 15–18 °C temperature range only, when Q$_{10\,dev}$ rapidly increased (reached a value double that for the interval 12–15 °C), then Q$_{10\,dev}$ gradually decreased again. The extents of Q$_{10\,dev}$ and Q$_{10\,gr}$ acceleration for the corresponding intervals were comparable. Both development and growth were most distinctively accelerated within the interval 9–12 °C (Q$_{10}$ reaching multifold differences relative to all other temperature intervals).

## 3.2.  SURVIVAL

The accumulated survival rate for the embryonic period (i.e. survival from Fe to H$_{50}$) at 9–24 °C ranged from 52.50 to 73.18% on average, depending upon temperature. All eggs incubated at temperatures 27–36 °C died (survival decreased to 0%) within several hours, i.e. 1 h (36 °C), 3.75 h (33 °C), 8 h (30 °C) and 8.5 h (27 °C) after egg activation in the 4-blastomer (30–36 °C) and 8-blastomer (27 °C) stages (Table 2).

Survival during the larval period evaluated from the egg activation to any of three key events, i.e. to first exogenous food intake (S), absolute yolk sac depletion (Re) and 3–4 days after Re, varied in ranges of 24.95–70.76% (S), 17.71–69.18% (Re) and 0.00 – 66.53% (3–4 days after Re). All larvae held at 9 and 12 °C died (survival equal to 0%) with the passing of several days post-Re, i.e. 3 days (12 °C) or 4 days (9 °C) after Re (Table 2).

The accumulated survival rates differed significantly at all crucial events depending upon temperature, i.e. at: (1) H$_{50}$ (F$_{9,20}$ = 123.01, P < 0.01) when survival differed significantly (P < 0.05) at 27–36 °C from the  thers (these temperatures were then excluded from subsequent statistical calculations); (2) S (F$_{9,20}$ = 126.80, P < 0.01) when survival differed significantly (P < 0.05) at 9 °C only; (3) Re (F$_{9,20}$ = 137.00, P < 0.01); and (4) 3–4 days after Re (F$_{9,20}$ = 139.47, P < 0.01) when survival was significantly different  (P < 0.05) at both 9 and 12 °C (summarized in Figure 14).
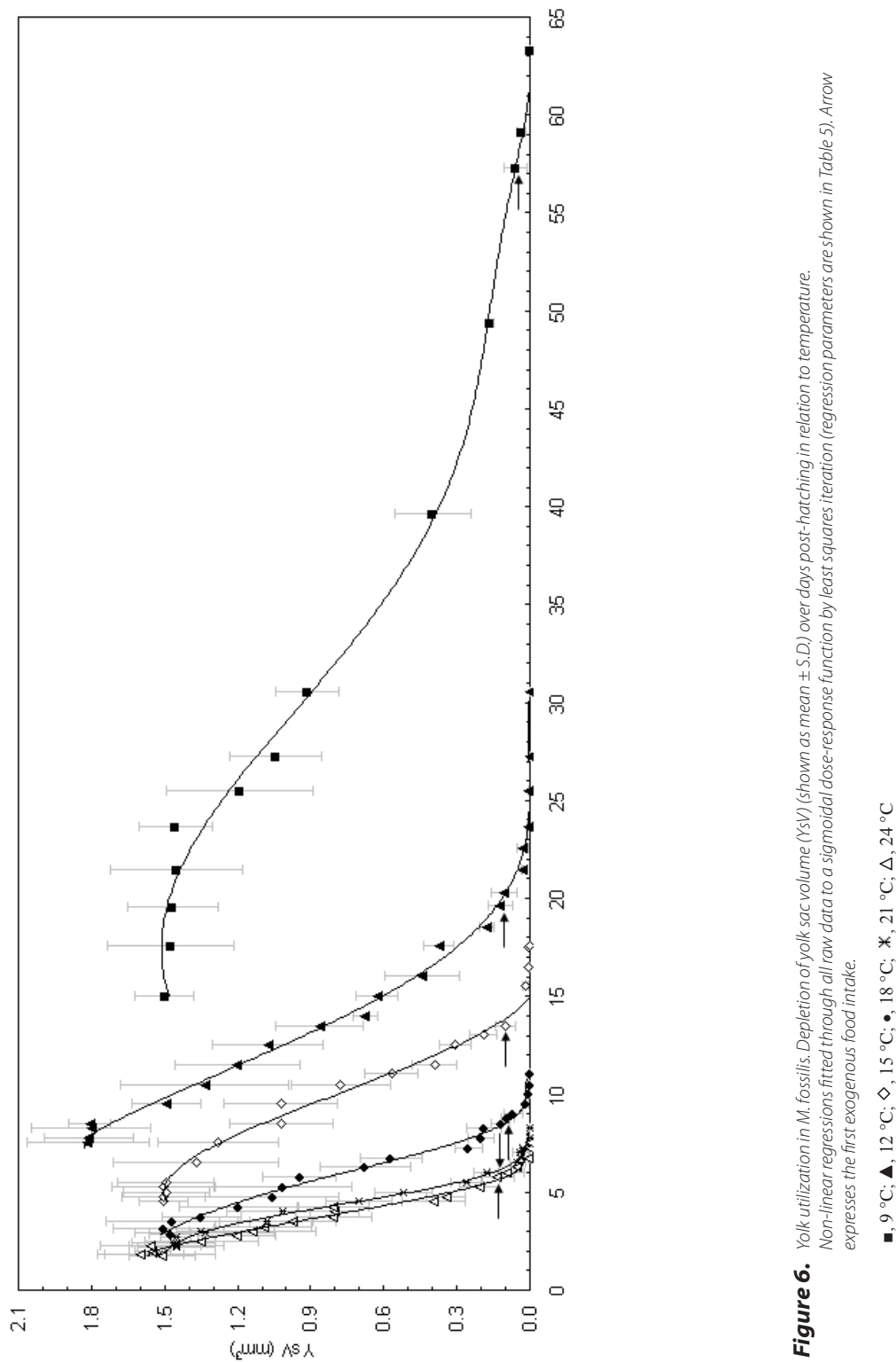
**Figure 6.** *Yolk utilization in M. fossilis. Depletion of yolk sac volume (YsV) (shown as mean ± S.D.) over days post-hatching in relation to temperature. Non-linear regressions fitted through all raw data to a sigmoidal dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*

■, 9 °C; ▲, 12 °C; ◇, 15 °C; ◆, 18 °C; ✶, 21 °C; △, 24 °C

**Table 5.** *Relationship between morphometric characteristics [total length ($L_T$), standard length ($L_S$), pre-anal length (prAn), post-anal length (psAn), pre-orbital length (prOrb), orbital length (Orb), yolk sac volume (YsV)] and temperature in M. fossilis. Non-linear regressions fitted by least squares iteration through all raw data to a logarithmic, polynomial or sigmoidal dose-response function. Data for $L_T$, $L_S$, prAn, psAn, prOrb and Orb were fitted by a logarithmic function ($y = a + b / \ln x$) (at 9 °C) or a polynomial function of the 5th order ($y = a + b \cdot x + c \cdot x^2 + d \cdot x^3 + e \cdot x^4 + f \cdot x^5$) (at 12–24 °C). Data for YsV were fitted by a sigmoidal function ($y = a + b / \{1 + e^{[-(x-c)/d]}\}$). All regressions were statistically significant ($P < 0.01$).*

*T, temperature (in °C); F, test criterion; N, number of observations; D.f., degrees of freedom; $r^2$, correlation coefficient*

| T (°C) | Characteristic | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|

**Total length ($L_T$)**

| T (°C) | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 241.70 | 122 | 1 | < 0.01 | 0.88 | 9.8015 | -15.5701 | | | | |
| 12 | 969.68 | 151 | 1 | < 0.01 | 0.90 | -2.7170 | 2.0044 | -0.2026 | 0.0107 | -0.0003 | $2.8330 \cdot 10^{-6}$ |
| 15 | 1167.87 | 120 | 1 | < 0.01 | 0.92 | -8.7165 | 6.2415 | -1.1503 | 0.1078 | -0.0049 | 0.0001 |
| 18 | 1323.04 | 130 | 1 | < 0.01 | 0.92 | 1.4921 | 0.8672 | 0.1548 | -0.0651 | 0.0073 | -0.0003 |
| 21 | 1152.32 | 115 | 1 | < 0.01 | 0.93 | 0.1609 | 3.6023 | -1.2096 | 0.2512 | -0.0272 | 0.0012 |
| 24 | 830.86 | 134 | 1 | < 0.01 | 0.92 | 3.7879 | -0.7745 | 0.9576 | -0.2709 | 0.0321 | -0.0013 |

**Standard length ($L_S$)**

| T (°C) | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 170.03 | 122 | 1 | < 0.01 | 0.70 | 8.5198 | -12.3987 | | | | |
| 12 | 746.52 | 151 | 1 | < 0.01 | 0.87 | -2.2144 | 1.8961 | -0.2005 | 0.0108 | -0.0003 | $2.8501 \cdot 10^{-6}$ |
| 15 | 977.59 | 120 | 1 | < 0.01 | 0.91 | -8.1860 | 6.0013 | -1.1106 | 0.1028 | -0.0046 | 0.0001 |
| 18 | 1068.01 | 130 | 1 | < 0.01 | 0.90 | 1.2474 | 1.1603 | 0.0309 | -0.0488 | 0.0064 | -0.0003 |
| 21 | 884.41 | 115 | 1 | < 0.01 | 0.91 | -0.1797 | 4.2215 | -1.5997 | 0.3387 | -0.0359 | 0.0015 |
| 24 | 717.41 | 134 | 1 | < 0.01 | 0.91 | 4.6540 | -1.9029 | 1.4750 | -0.4070 | 0.0496 | -0.0022 |

**Pre-anal length (prAn)**

| T (°C) | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 109.42 | 122 | 1 | < 0.01 | 0.71 | 5.7330 | -6.6630 | | | | |
| 12 | 409.65 | 151 | 1 | < 0.01 | 0.74 | -0.3109 | 1.2118 | -0.1330 | 0.0072 | -0.0002 | $1.8951 \cdot 10^{-6}$ |
| 15 | 438.79 | 120 | 1 | < 0.01 | 0.80 | -4.3840 | 3.9874 | -0.7435 | 0.0673 | -0.0029 | $4.8810 \cdot 10^{-5}$ |
| 18 | 559.84 | 130 | 1 | < 0.01 | 0.82 | 3.3996 | -0.3535 | 0.2891 | -0.0656 | 0.0064 | -0.0002 |
| 21 | 491.61 | 115 | 1 | < 0.01 | 0.83 | 0.2039 | 3.6883 | -1.5873 | 0.3550 | -0.0384 | 0.0016 |
| 24 | 420.66 | 134 | 1 | < 0.01 | 0.86 | 6.4724 | -4.5424 | 2.4902 | -0.6037 | 0.0681 | -0.0029 |

**Post-anal length (psAn)**

| T (°C) | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 381.36 | 122 | 1 | < 0.01 | 0.85 | 2.7800 | -5.7021 | | | | |
| 12 | 1342.76 | 151 | 1 | < 0.01 | 0.95 | -4.8308 | 1.6739 | -0.1925 | 0.0110 | -0.0003 | $3.1861 \cdot 10^{-6}$ |
| 15 | 1423.27 | 120 | 1 | < 0.01 | 0.96 | -7.5814 | 3.2430 | -0.5535 | 0.0470 | -0.0020 | $3.1789 \cdot 10^{-5}$ |
| 18 | 1652.79 | 130 | 1 | < 0.01 | 0.96 | -1.6249 | 1.1817 | -0.1694 | 0.0045 | 0.0009 | -0.0001 |
| 21 | 1537.08 | 115 | 1 | < 0.01 | 0.97 | -1.8362 | 2.4638 | -0.9471 | 0.1935 | -0.0196 | 0.0008 |
| 24 | 722.95 | 134 | 1 | < 0.01 | 0.95 | -1.0459 | 1.7086 | -0.6316 | 0.1327 | -0.0147 | 0.0007 |

**Table 5.** *continuation*

| | Pre-orbital length (prOrb) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
| 9 | 409.98 | 122 | 1 | < 0.01 | 0.86 | 0.6652 | -1.5814 | | | | |
| 12 | 795.53 | 151 | 1 | < 0.01 | 0.86 | -1.2319 | 0.3923 | -0.0426 | 0.0022 | -0.0001 | $5.1252.10^{-7}$ |
| 15 | 571.68 | 120 | 1 | < 0.01 | 0.87 | -1.1365 | 0.5562 | -0.0920 | 0.0072 | -0.0003 | $3.4569.10^{-6}$ |
| 18 | 638.89 | 130 | 1 | < 0.01 | 0.88 | -0.1736 | -0.0482 | 0.1001 | -0.0264 | 0.0027 | -0.0001 |
| 21 | 686.89 | 115 | 1 | < 0.01 | 0.90 | -0.1643 | 0.2657 | -0.0835 | 0.0132 | -0.0009 | $1.7371.10^{-5}$ |
| 24 | 621.19 | 134 | 1 | <0.01 | 0.89 | -0.5202 | 0.7260 | -0.2833 | 0.0515 | -0.0041 | 0.0001 |

| | Orbital length (Orb) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
| 9 | 301.38 | 122 | 1 | < 0.01 | 0.77 | 0.4418 | -0.6546 | | | | |
| 12 | 486.92 | 151 | 1 | < 0.01 | 0.80 | -1.1919 | 0.4357 | -0.0500 | 0.0027 | -0.0001 | $7.2592.10^{-7}$ |
| 15 | 455.28 | 120 | 1 | < 0.01 | 0.85 | -2.3989 | 1.3085 | -0.2488 | 0.0227 | -0.0010 | $1.6788.10^{-5}$ |
| 18 | 454.59 | 130 | 1 | < 0.01 | 0.82 | 0.3284 | -0.1387 | 0.0581 | -0.0103 | 0.0009 | $-2.6557.10^{-5}$ |
| 21 | 578.42 | 115 | 1 | < 0.01 | 0.89 | -0.3655 | 0.5348 | -0.1905 | 0.0352 | -0.0032 | 0.0001 |
| 24 | 559.26 | 134 | 1 | < 0.01 | 0.88 | 1.9960 | -2.1151 | 0.9449 | -0.1985 | 0.0200 | -0.0008 |

| | Yolk sac volume (YsV) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F | N | D.f. | P | $r^2$ | a | b | c | d | e | f |
| 9 | 978.41 | 122 | 1 | < 0.01 | 0.94 | 0.0174 | 1.6097 | 32.2718 | -6.1227 | | |
| 12 | 597.00 | 151 | 1 | < 0.01 | 0.96 | -0.0195 | 2.2889 | 11.9183 | -3.0064 | | |
| 15 | 989.95 | 120 | 1 | < 0.01 | 0.93 | -0.0566 | 1.5994 | 10.2770 | -1.5865 | | |
| 18 | 1198.77 | 130 | 1 | < 0.01 | 0.94 | -0.0597 | 1.7241 | 5.8225 | -1.2788 | | |
| 21 | 1410.80 | 115 | 1 | < 0.01 | 0.96 | -0.0222 | 1.5748 | 4.4038 | -0.7849 | | |
| 24 | 678.94 | 134 | 1 | < 0.01 | 0.93 | -0.0529 | 1.8228 | 3.7065 | -0.9436 | | |

# 4. DISCUSSION

## 4.1. DEVELOPMENT

Temperatures within the presumed optimum range (15–24 and 15–21 °C for embryonic and larval periods, respectively; see below) accelerate the development of *M. fossilis* in terms of incubation period duration, hatching period duration, and time of Ac, S, Re observation during different intervals of the embryonic and larval periods in a uniform (inversely proportional) way, as is the case for common carp *Cyprinus carpio* L. (Penaz et al., 1983), vendace *Coregonus albula* (L.) (Luczyński and Kirklewska, 1984) and sea trout *Salmo trutta trutta* L. (Raciborski, 1987).

In *M. fossilis* embryos, the threshold temperature ($t_0$) and effective day-degrees ($D°_{eff}$) are 8.36 °C and 28.74, respectively. Thus, within Kamler's (2002) classification of temperature requirements for embryonic development considering the relationship between $t_0$ and $D°_{eff}$, *M. fossilis* occupies an intermediate, ambivalent position near that of phytophilous cyprinids spawning like weatherfish in stagnant waters during late spring and early summer (Kottelat and Freyhof, 2007), i.e.: (1) in $t_0$ terms, comparable to lake minnow *Eupallasella percnurus* (Pallas) ($t_0$ = 8.30 °C, $D°_{eff}$ = 45.50; Kamiński et al., 2006) and bream *Abramis brama* (L.) ($t_0$ = 8.60 °C, $D°_{eff}$ = 41.00; Kamler, 2002), and (2) in $D°_{eff}$ terms, *C. carpio* ($t_0$ = 9.90 or 11.10 °C, $D°_{eff}$ = 30.00 or 34.00; Kamler, 2002), tench *Tinca tinca* (L.) ($t_0$ = 11.70 °C, $D°_{eff}$ = 29.00; Kamler, 2002) or the nearby lithophilous species, vimba *Vimba vimba* (L.) ($t_0$ = 10.10 °C, $D°_{eff}$ = 28.00; Kamler, 2002).

Available evidence as to the effect of temperature upon hatching size in ray-finned fishes is

inconsistent and contradictory. Larval size at hatch commonly is inversely related to temperature (lower temperatures produce bigger larvae), i.e. hatching is generally considered an age-related rather than size-related event (Penaz et al., 1983; Kamler et al., 1994, 1998; Ojanguren and Braña, 2003; Martell et al., 2005). Contrary to the preceding, Alderdice and Forrester (1974) and Pepin et al. (1997), respectively, reported on a significant decline in size of freshly hatched flathead sole *Hippoglossoides elassodon* Jordan and Gilbert and Atlantic cod *Gadus morhua* L. larvae with decreasing temperature (in the latter, an effect of increased metabolic requirements at temperatures close to the lower boundary of thermal tolerance range is supposed). However, as in Atlantic herring *Clupea harengus* L. (Blaxter and Hempel, 1963), Black Sea salmon *Salmo labrax* Pallas (Zalicheva, 1981), haddock *Melanogrammus aeglifinus* (L.) (at 2–8 °C, Martell et al., 2005) and *G. morhua* (only batch 2, Jordaan et al., 2006), size of newly hatched *M. fossilis* larvae is neither positively nor negatively correlated with temperature ($L_T$ of larvae at hatch does not significantly differ based upon temperature except at 12 °C; see comments in Results). In addition, it may be supposed that (1) length thus might determine the age at hatching, rather than the age at hatching is determining the hatching length in *M. fossilis*; and (2) growth within eggs is most likely unequal, and thus a synergistic effect of both embryonic growth and temperature probably determines the hatching time in *M. fossilis* (at least within the temperature range studied) (Drozd et al., 2009).

Developmental stage of freshly hatched larvae commonly is negatively related to incubation temperature (Penaz et al., 1983; Ojanguren and Braña, 2003), i.e. hatching event is only loosely linked to a specific developmental stage in fishes (Yamagami, 1988). Nevertheless, developmental stage of newly hatched *M. fossilis* larvae shows only a slight declining tendency upon rising temperature [stage 37 at 9–18 °C and stage 36 at 21–24 °C; Kostomarova's (1975)]. This may not be explained, however, through other water condition (especially oxygen concentration), as in Kotlyarevskaja (1967), because oxygen saturation was approximately 90% for each temperature treatment in the present study.

According to Kamler's (2002) criteria, no apparent influence of incubation temperature upon either size or developmental stage of newly hatched *M. fossilis* larvae may be evidence of a very wide zone of viable temperatures for *M. fossilis* – at least during the embryonic period (from Fe to H).

The size of *M. fossilis* larvae at the onset of mixed (S), as well as exogenous (Re), feeding was found to be independent of temperature (except at 9 °C), as in the cases of yellowtail flounder *Limanda ferruginea* (Storer) (Howell, 1980), summer flounder *Paralichthys dentatus* (L.) (Johns et al., 1981), turbot *Psetta maxima* (L.) (Quantz, 1985), *C. nasus* (Kamler et al., 1998), *C. harengus* (Geffen, 2002), and *M. aeglefinus* (Martell et al., 2005). The developmental stage of larvae at Re can be considered temperature independent in *M. fossilis* as well.

*M. fossilis* inhabits waters often suffering temperature, pH and water level disturbances, as well as high ammonium and sulphate but low oxygen concentrations (Meyer and Hinrichs, 2000; Pekarik et al., 2008). That is to say, their localities generally represent unfavourable and ever-changing environments. Weatherfish larvae hatch from sticky eggs attached to underwater vegetation and hang fast to plants by the head for a couple of days (Grieb, 1937; Kryzanovskij, 1949; Kostomarova, 1975). Consequently, the similar size and developmental stage of *M. fossilis* larvae at every key event – i.e. at hatching ($H_{50}$), as well as at the onset of mixed (S) and exogenous (Re, yolk sac full depleted) feeding – regardless of temperature may be perceived as either a functional compromise between ontogenetic and behavioural traits conditioned by both endogenous and exogenous (abiotic and biotic) factors or an outcome from a set of various anatomical, physiological and developmental adaptations conducive to successful reproduction and survival in such environmental conditions.

Upon hatching, and for somewhat different reasons, both immature larvae (at higher temperatures, i.e. at 21–24 °C in present study) and overly mature larvae (at lower temperatures, i.e. 9–12 °C in present study) must not leave the eggs. Immature, immobile individuals face a risk of early death after hatching

into life-inhibiting conditions, as the essential, additional respiratory organs will develop later [the first one, the outer filamentous gills, develops at Kostomarova's (1975) stage 38]. Also at risk are over-mature individuals close to full yolk sac depletion, which have low energy resources stored in the yolk sac as a result of their prolonged time spent in the egg at lower temperatures. After hatching, they can face starvation leading quickly to death [according to Kamler (1992)].

From the viewpoint of predetermined larval size at hatching, the biotic interactions – in particular interspecific food competition and predation (Wootton, 1990; Kamler, 2002) – may be as significant as are the abiotic environmental conditions (Keckeis et al., 1996; Prokes et al., 1998; Schiemer et al., 2003) and parental attributes (Panagiotaki and Geffen, 1992; Kamler, 2008). This is because these conditions and attributes determine initial food size during the transition to both mixed and exogenous feeding (larger, better developed larvae have a greater chance to succeed in the interspecific food competition), and these further determine the size of predator able to utilize the particular developmental stages (immature larvae are more vulnerable to predation) (Wootton, 1990). Prospects for greater survival rates in larvae might be improved by rapid growth and by variation in behaviour (Eeton and DiDomenico, 1986; Webb and Weihs, 1986) associated with continually increasing maximum and mean escape speed (Green and Fisher, 2004) during the first couple days after hatching. Moreover, it looks like in *M. fossilis* "something specific and new" is required for transition to exogenous nutrition and thus to colonization and utilization of new microhabitats and food resources. [Kotlyarevskaja (1967) observed weatherfish larvae actively climbing over aquatic vegetation in search of suitable food.] *M. fossilis* larvae at the onset of exogenous feeding achieve that of Kryzanovskij's (1949) stages where they possess rudimentary outer filamentous gills, but also well-developed segmental blood vessels in the dorsal and anal parts of the fin-fold, as well as in pectoral fins, and considerably enlarged peri-intestinal blood vessels. Consequently, and regardless of temperature, *M. fossilis* larvae do not fully deplete the yolk mass, i.e. they do not start to utilize the exogenous nutrition only, until the gut becomes one of the functional additional respiratory organs. In addition to those attributes mentioned above, chances of survival and reproduction at the typical natural localities where *M. fossilis* occurs might be amplified by a fraction spawning strategy, as Suzuki (1983) and Bohlen (1999b) reported for the related species *M. anguillicaudatus* and spined loach *Cobitis taenia* (sensu L.), respectively. To date, however, there are no data of that kind for *M. fossilis*.

The present study revealed that the two-stage curve (Kramer and Zweifel, 1970) fitted by polynomial function might provide a useful model for both growth (expressed as $L_T$ increase in time) as well as for the course in time of the standard, pre-anal, post-anal, pre-orbital and orbital lengths for *M. fossilis* larvae cultivated over a wide temperature range. At 9 °C, however, this rule is violated (see increased metabolic requirements below). In *M. fossilis*, as in fishes generally (Kamler, 1992, 2002), a period of moderate increase in size shortly after hatching is followed by a phase of minimal growth and then by a period of rapid growth that comes soon after the onset of mixed feeding (S) (corresponding to a notable inflection region in the course of all morphometric characteristics studied, see Figures 8–13). In orbital length, however, a course inflection point is moved backwards, i.e. the eyes as one of the sensory organs accelerate their development and start rapidly to grow relatively long before the onset of mixed feeding itself. This probably is a response to environmental needs, and especially timely food detection (Ibrahim et al., 2006; Mukai et al., 2008) and predator avoidance (Fuiman, 1994; Poling and Fuiman, 1997).

The duration of yolk mass depletion typically declines with rising temperature within the entire viable temperature range (summarized by Kamler, 1992, 2008). However, no effect of increasing water saturation within body tissues (including the yolk mass as well) by decreasing temperatures during early ontogeny, as supposed e.g. by Kamiński et al. (2006) in *E. percnurus*, is confirmed in *M. fossilis* over the larval period (as there was no statistical difference in yolk sac volume among temperatures except for at 12 °C; see Table 2).

In an interfamilial comparison, cobitids – *M. fossilis* (ca 4–6% of Y.r.) and flatfishes – *P. maxima* (ca 8% of Y.r. – Quantz, 1985) have available very low energy reserves deposited in the yolk sac at the onset of mixed feeding (S) compared to salmonids – Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (ca 20% of Y.r. – Heming, 1982) and *S. trutta trutta* (ca 55% of Y.r. – Raciborski, 1987) or centrarchids – largemouth bass *Micropterus salmoides* (Lacepède) (ca 21% of Y.r. – Laurence, 1969). In addition, Ito and Suzuki (1977) observed in the related species *M. anguillicaudatus* that larvae fed on detritus only and ingested no planktonic organisms. From an evolutionary viewpoint, both of these facts might support a presumption of relatively low dietary specialization and demands of loach larvae (according with a certain guarantee of finding sufficient suitable food regardless of abiotic factors actually existing at the locality of occurrence). To date, however, nothing is known about the actual dietary requirements or foraging behaviour of *M. fossilis* during early life history.

During the endogenous feeding period from hatching to the onset of mixed feeding (H–S), *M. fossilis* converted more of its yolk mass to body tissue (as expressed by the ratio of total length achieved and undigested yolk sac reserves) as incubation temperature was decreasing. This is comparable to tautog *Tautoga onitis* (L.) (Laurence, 1973), Atlantic salmon *Salmo salar* L. (Hamor and Garside, 1977) and *O.tshawytscha* (Rombough, 1985). In addition, according to Kokurewicz (1969), Alderdice et al. (1988) and Kamler (1992), an optimum temperature in terms of yolk mass efficiency occurred in *M. fossilis* at he intermediate (12–15 °C), i.e. not at the highest, viable temperatures. At temperatures above and below this range, the metabolic requirements are proportionally greater than those contributing to tissue production. This results in retarded growth (Johns et al., 1981; Blaxter, 1992; Kamler, 1992; Pepin et al., 1997) and thus in noticeably smaller larvae (see Table 2, Figure 7). On this basis, and combined with a low survival rate (Table 2, Figure 14), 9 °C should be considered a temperature lying already close to the lower boundary of the thermal tolerance range for the early larval stages of *M. fossilis*.

In general terms, yolk mass conversion efficiency from egg activation to absolute yolk sac depletion (expressed in terms of final larval total length) increases with rising temperature within the low temperature range (9–12 °C), but within the circum-(sub)optimal temperature range (12–24 °C) no apparent effect of temperature upon yolk sac utilization efficiency was observed. This is similar to observations regarding *C. harengus* (range: 3.5–17 °C; Overnell, 1997) and *C. nasus* (range: 10–19 °C; Kamler et al., 1998).

A general pattern of $Q_{10}$ declining with rising temperature over the entire early life history (summarized by Kamler, 2002) was highlighted in the *M. fossilis* embryonic period by a markedly elevated $Q_{10\,dev}$ value within the 9–12 °C range (triple the $Q_{10\,dev}$ value for the adjacent interval of higher temperature), as well as by an apparent violation of this common pattern observed for developmental rate within the range 15–18 °C ($Q_{10\,dev}$ reached twice the value for the adjacent interval of lower temperature). Both of these facts may be useful in determining presumable temperature requirements for *M. fossilis* embryonic development. The former probably evidences a shift from a zone of temperatures close to lethality to one of suboptimal temperatures and the latter to an onset of optimal temperatures, which is also supported by limitation upon the rate of developmental progress ($Q_{10\,dev} = 1$) in the interval 21–24 °C.

Based on comparison of $Q_{10}$ values for developmental rate ($Q_{10\,dev}$) and growth rate ($Q_{10\,gr}$), it might be assumed that the ontogeny and growth of *M. fossilis*, like those of African catfish *Clarias gariepinus* (Burchell) (Kamler et al., 1994) and *C. nasus* (Kamler et al., 1998), are accelerated by temperature in a similar way (including the comparable values for both coefficients in the range of 15–24 °C) over the entire period of larval endogenous feeding. Only the extents of $Q_{10\,dev}$ and $Q_{10\,gr}$ acceleration within temperature ranges 9–12 and 12–15 °C differ from one another, thus suggesting a stronger enhancement of growth compared to development during transition to the temperature conditions suboptimal and optimal for larval development.
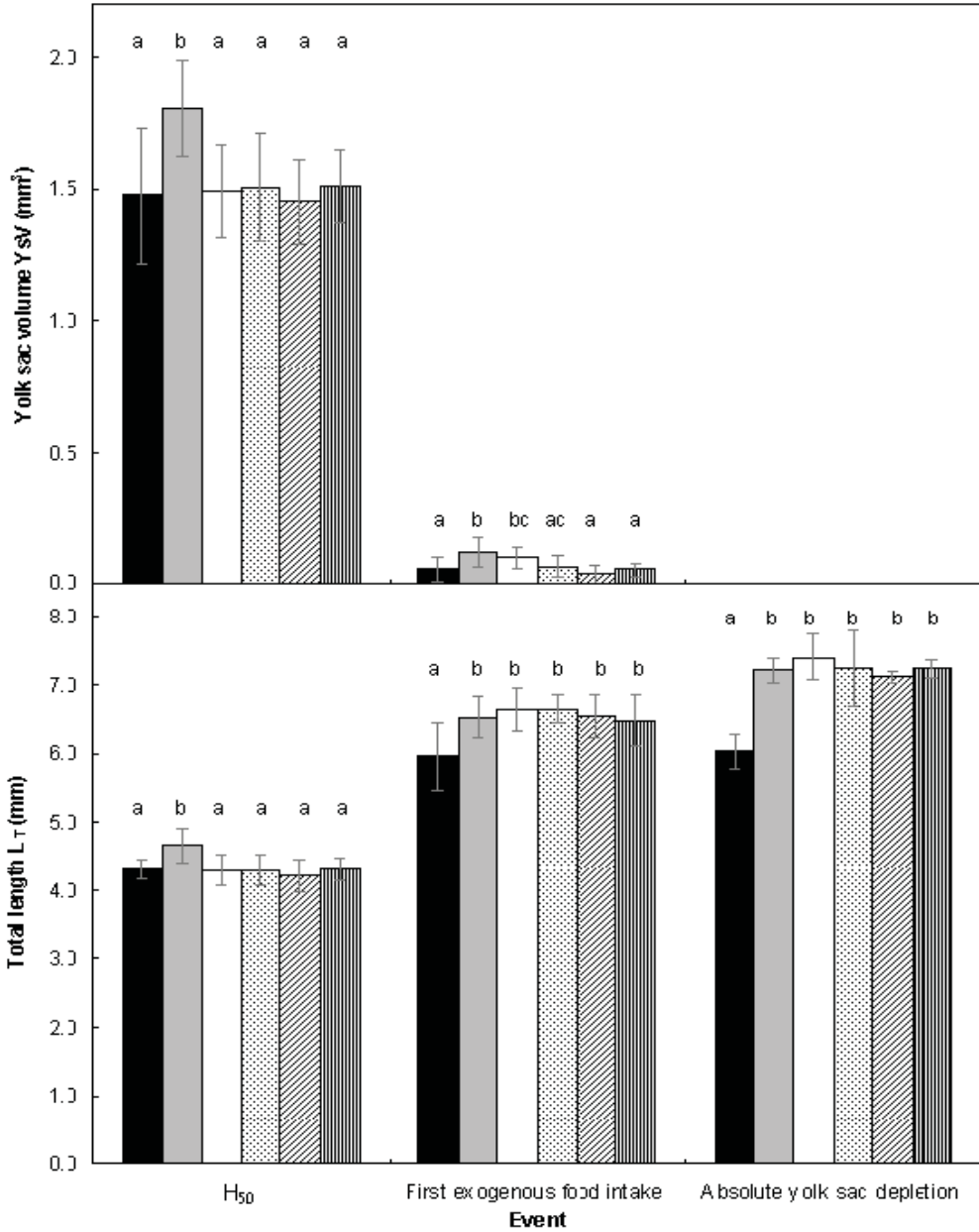
**Figure 7.** *Yolk sac volume (YsV; mm³) and total length ($L_T$; mm) in relation to temperature (shown as mean ± S.D.) during early ontogeny of M. fossilis. Both, YsV and $L_T$ were evaluated at three key ontogenetic events, i.e. at $H_{50}$, first exogenous food intake and/or absolute yolk sac depletion.*
*Groups with the same superscript (a, b, c) do not significantly differ (Tukey HSD test, P = 0.05).*
*■, 9 °C; ▨, 12 °C; □, 15 °C; ▨, 18 °C; ▨, 21 °C; ▥, 24 °C; $H_{50}$ point of hatching of 50% of individuals*
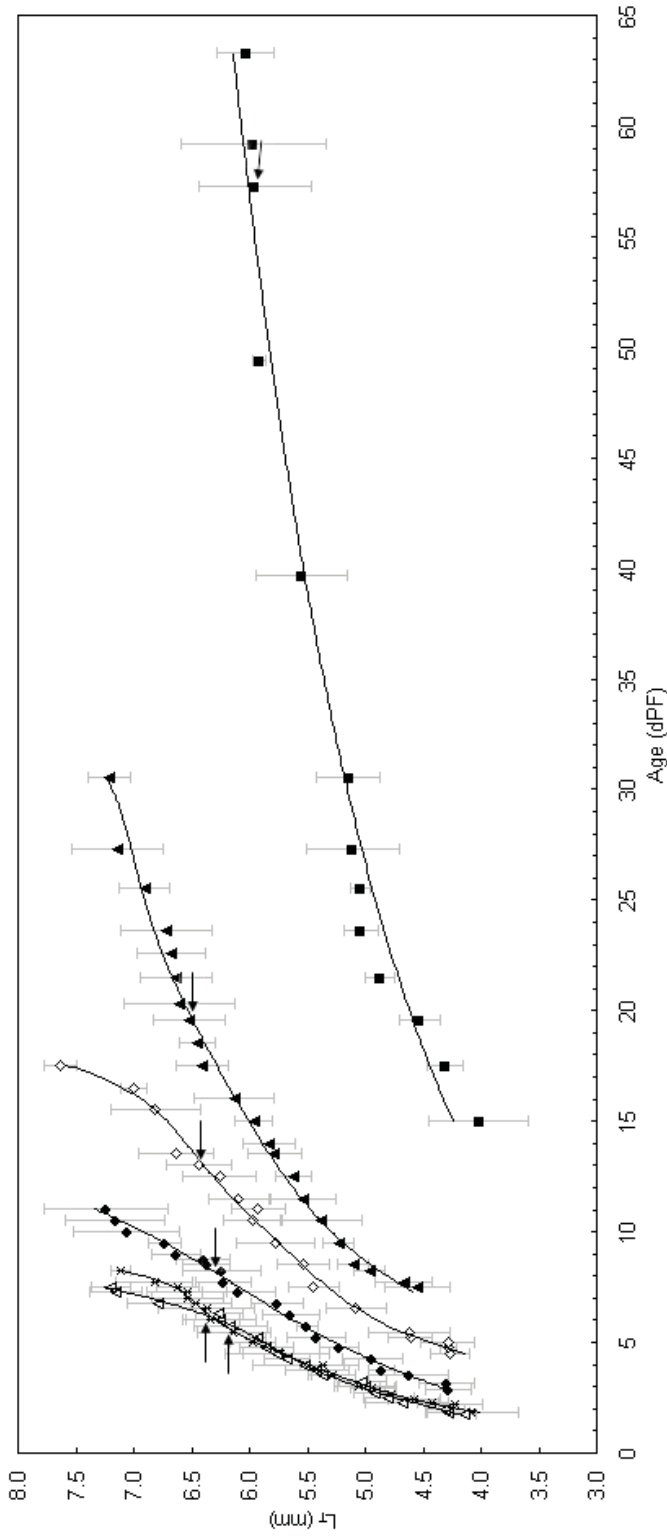
**Figure 8.** *Increase of total length ($L_T$) (shown as mean ± S.D.) over days post-hatching in relation to temperature in M. fossilis. Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*

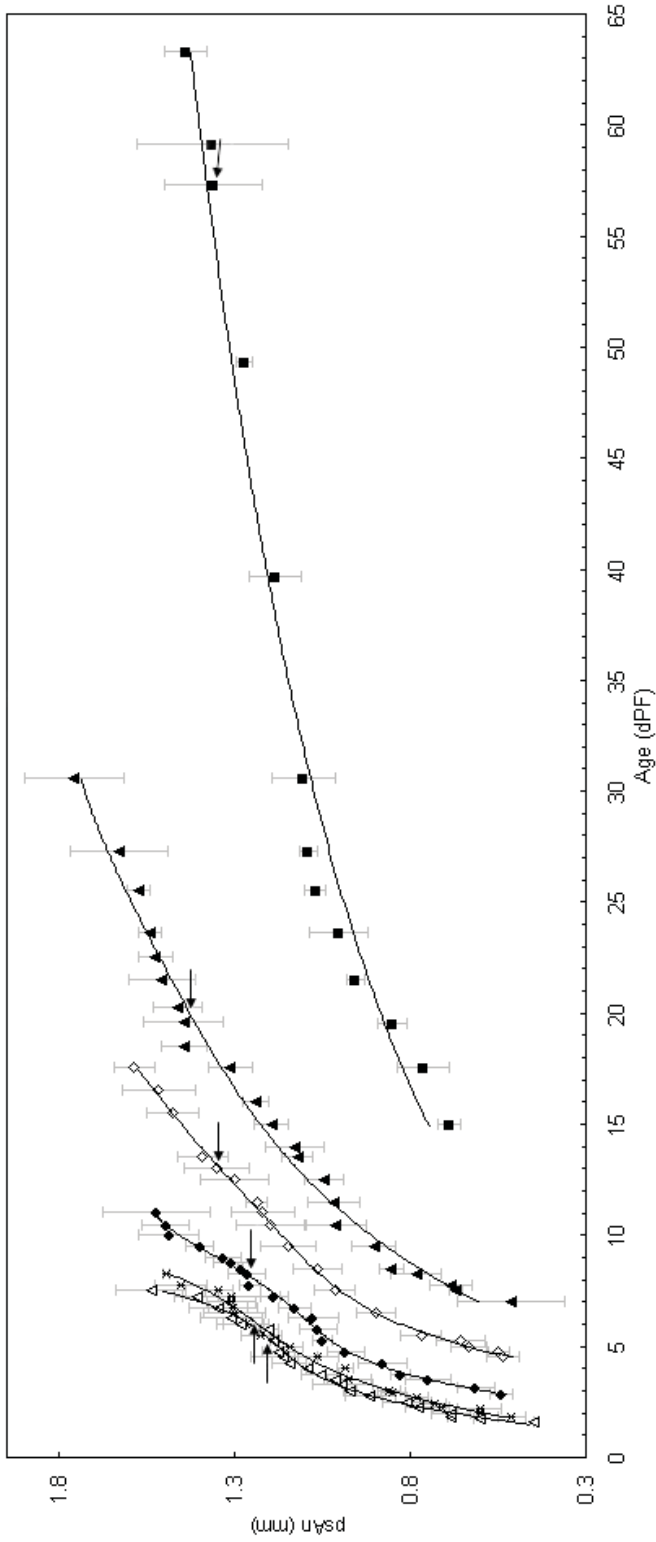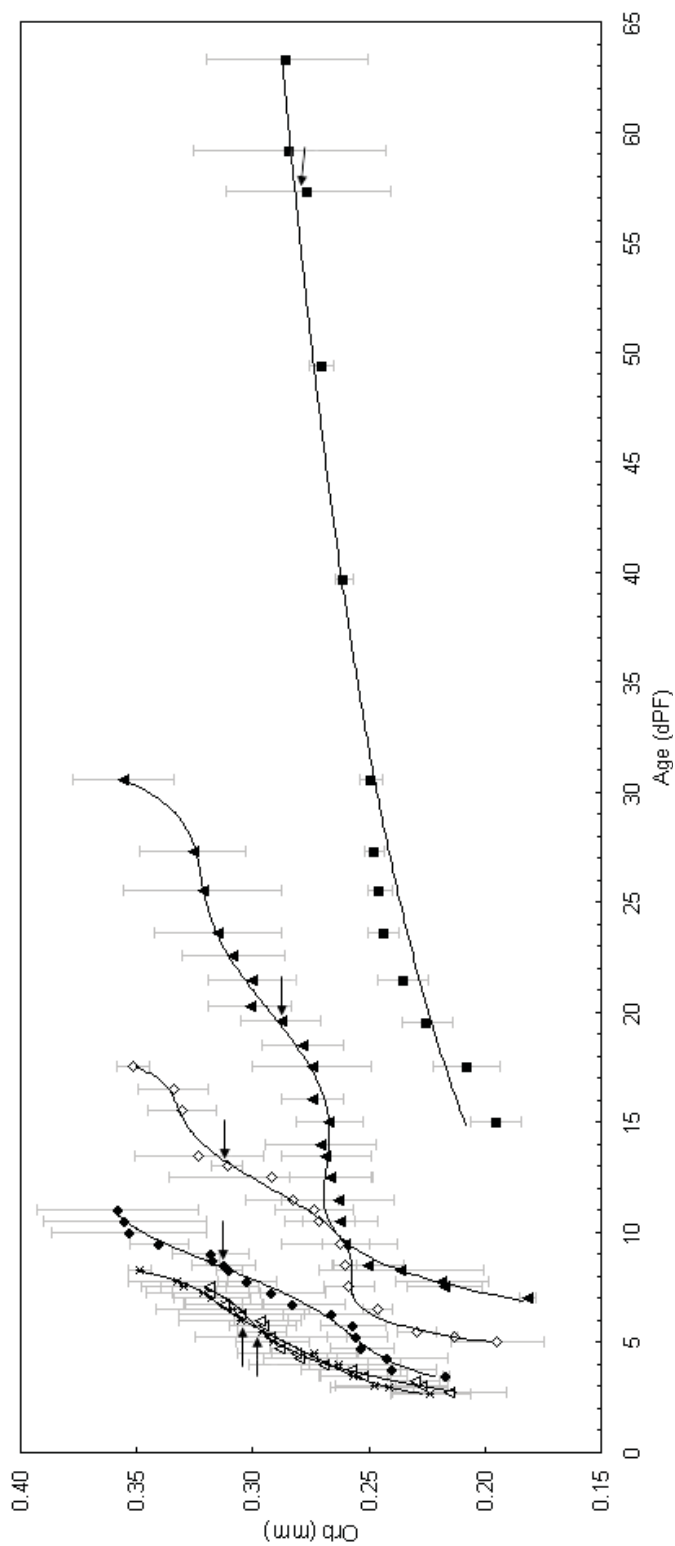*■, 9 °C; ▲, 12 °C; ◇, 15 °C; ●, 18 °C; ✳, 21 °C; △, 24 °C*
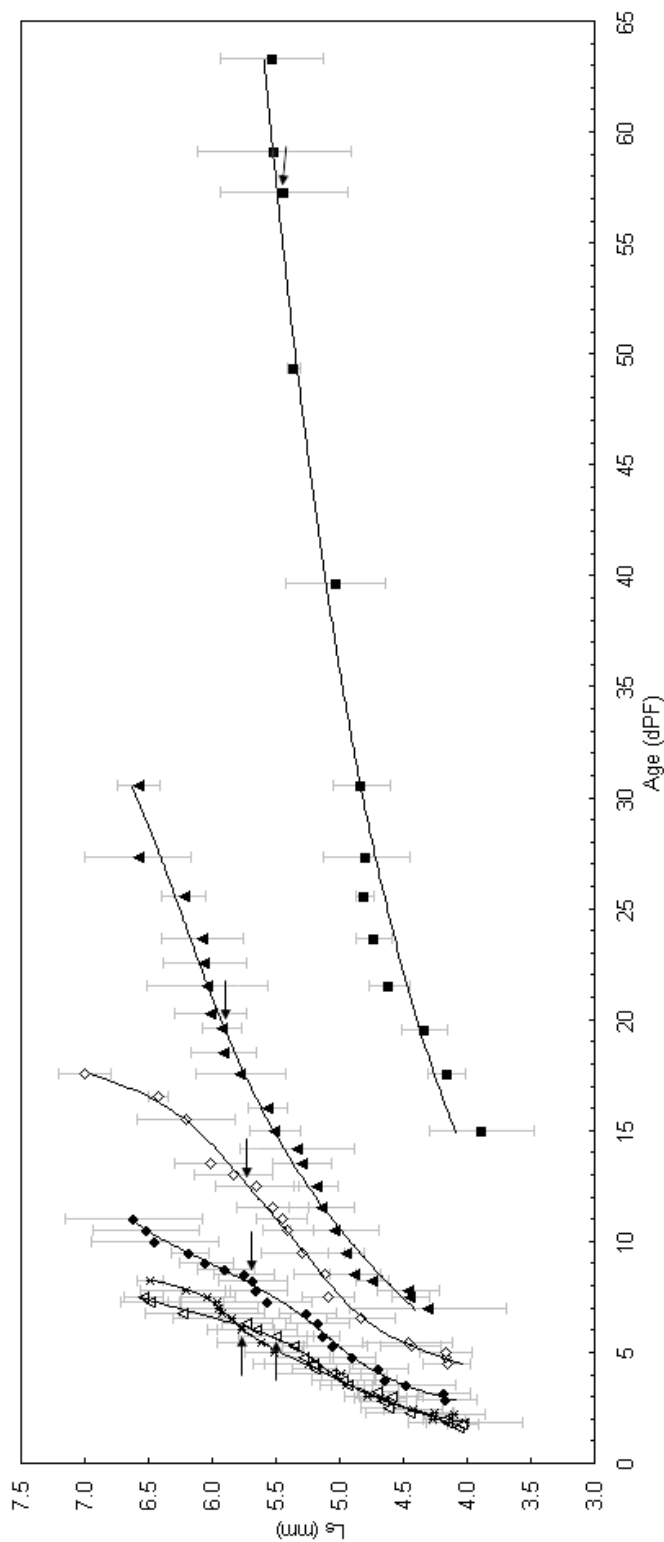
**Figure 9.** *Increase of standard length ($L_s$) (shown as mean ± S.D.) over days post-hatching in relation to temperature in M. fossilis. Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*
*■, 9 °C; ▲, 12 °C; ◇, 15 °C; ◆, 18 °C; ✷, 21 °C; △, 24 °C*
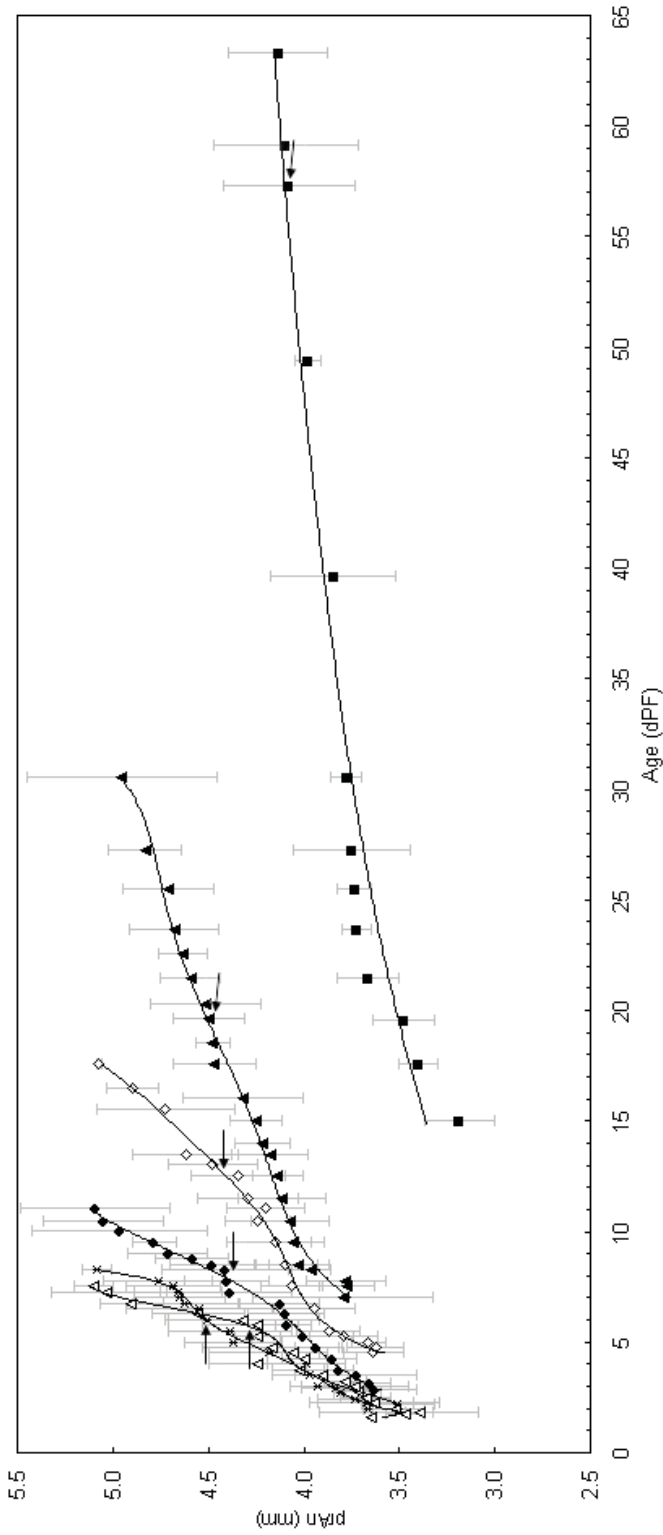
**Figure 10.** *Development of pre-anal length (prAn) over days post-hatching in relation to temperature in M. fossilis. Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*

■, 9 °C; ▲, 12 °C; ◇, 15 °C; ●, 18 °C; ✖, 21 °C; △, 24 °C
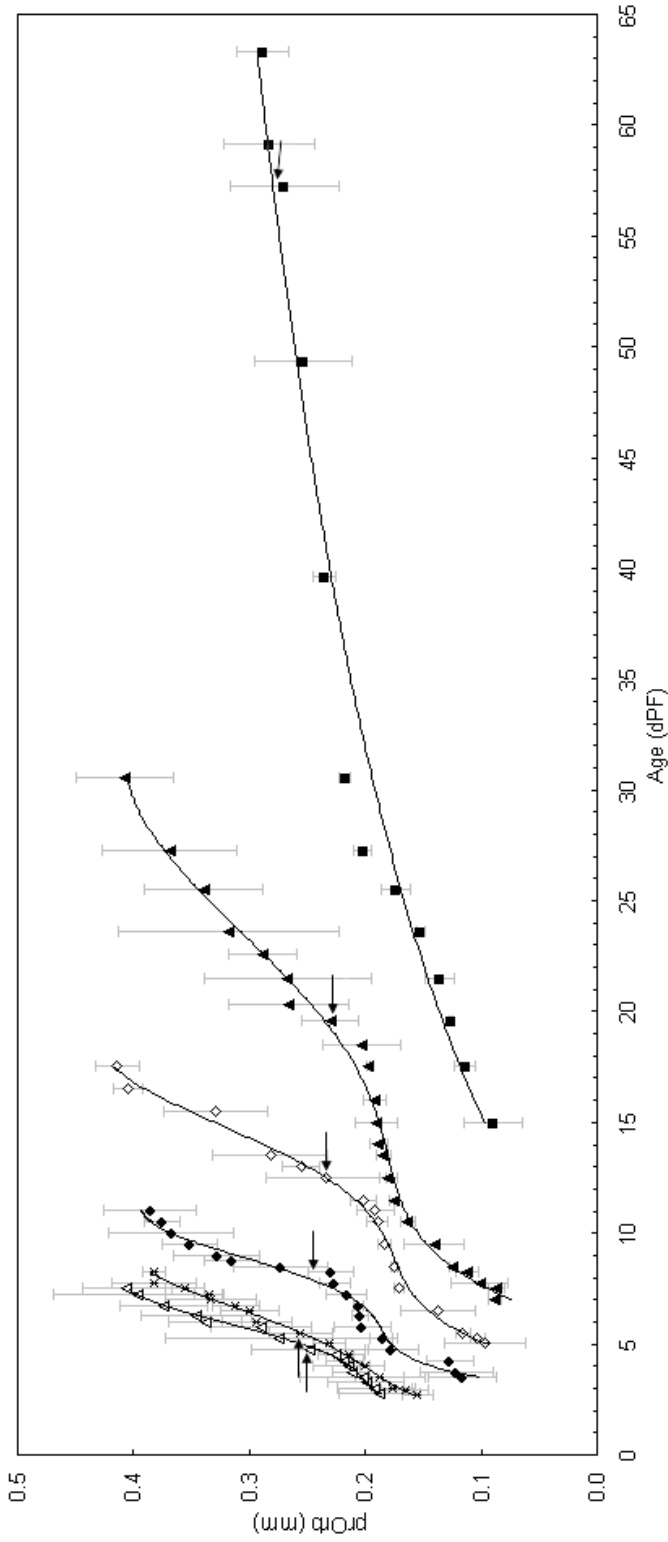
***Figure 11.*** *Development of pre-orbital length (prOrb) over days post-hatching in relation to temperature in M. fossilis.*
*Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters*
*are shown in Table 5). Arrow expresses the first exogenous food intake.*
*■, 9 °C; ▲, 12 °C; ◇, 15 °C; ◆, 18 °C; ✳, 21 °C; △, 24 °C*

**Figure 12.** *Development of post-anal length (psAn) over days post-hatching in relation to temperature in M. fossilis. Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*

*■, 9 °C; ▲, 12 °C; ◇, 15 °C; ●, 18 °C; ✳, 21 °C; △, 24 °C*

**Figure 13.** *Development of orbital length (Orb) over days post-hatching in relation to temperature in M. fossilis. Non-linear regressions fitted through all raw data to a logarithmic (9 °C) and polynomial (12–24 °C) dose-response function by least squares iteration (regression parameters are shown in Table 5). Arrow expresses the first exogenous food intake.*

■, 9 °C; ▲, 12 °C; ◇, 15 °C; •, 18 °C; ✴, 21 °C; △, 24 °C

## 4.2. SURVIVAL

Based on previous (Drozd et al., 2009) and present data on *M. fossilis* eggs and larvae incubated at stable temperatures through the entire early ontogeny, which have shown remarkably different effects of temperature on the particular stages of early ontogeny (especially in terms of survival), it is highly recommended to distinguish and separately evaluate the impacts of a wide temperature range on the embryonic and larval periods.

In *M. fossilis*, the zone of tolerated temperatures for the embryonic period (Fe–H) lies between ca 9 °C (development is theoretically arrested at ca 8.50 °C; the lowest viable temperature was mathematically estimated at ca 9.10 °C) and ca 24 °C. An assumed upper lethal temperature lies in the 24–27 °C range, when survival rapidly declines from an above-average value (ca 70%) to 0%. Temperatures above 24 °C (i.e. 27–36 °C in the present study) are thus believed to be lethal due to a complete life-inhibiting effect already during the embryonic period. A thermal optimum [i.e. survival over 60% according to criteria of Kostomarova (1975), Penaz et al. (1983) and Ozernyuk et al. (1987)] for *M. fossilis* embryonic development suggested by the present study to be in the range of 15–24 °C corresponds to thermal conditions similar to those observed in nature by both Grieb (1937) and Kryzanovskij (1949) during the spawning season of *M. fossilis*, as well as the optimal temperatures proposed by Kostomarova (1975) [(13)14–(20)24 °C], Alexeeva and Ozernyuk (1987) or Zdanovich et al. (2001) (14–22 °C) based on experiments in laboratory conditions. Temperatures under 15 °C should be considered suboptimal for the embryonic period (survival at 9 and 12 °C exceeded 50%). In an interspecific comparison, the zone of tolerated temperatures for the embryonic period in *M. fossilis* is shifted towards the lower temperatures compared to that in related loach species *M. anguillicaudatus*, in which Watanabe et al. (1948) observed normally proceeding hatching at 28–30 °C, and for which Kubota and Matsui (1955) and Wang et al. (2008), respectively, described the thermal optimum to be 25 °C and 25–27 °C. Nevertheless, 28 °C might be considered a critical or border-line temperature inducing sperm incorporation in . *anguillicaudatus* (Itono et al., 2007).

In *M. fossilis*, however, the larval period is characterized by continuous and even more enhanced influence of temperature on development in terms of survival as an effect of stable thermal condition over the entire study period (especially at lower temperatures), which is reflected in a narrower viable temperature range (15–24 °C) than could be expected based upon a theoretical assumption for the larval data set (development should be theoretically arrested at ca 7.00 °C; the lowest viable temperature was mathematically estimated at ca 7.80 °C) and the common pattern of thermal sensitivity of particular stages in early life history (larvae should tolerate a wider temperature range than do embryos, as summarized by Kamler, 2002).

Negative effects of lower temperatures (i.e. 9 °C and 12 °C) on *M. fossilis* larvae development in terms of survival (including also higher S.D. value – Table 2), apparent prolongation of yolk sac depletion, and reduced developmental and growth rates (especially at 9 °C) are visible during the entire post-hatching period. A real lethal impact of these temperatures is not expressed, however, until the onset of exogenous feeding (i.e. until absolute yolk sac depletion), when all individuals will die within the next few days (3 or 4 days post-Re). This might probably be attributed to poor food intake ability or insufficient ability to digest ingested food particles at lower temperatures leading up to complete energy exhaustion (in this study, however, there was no detailed examination of digestive enzymes secretion and activity). Generally, types, secretion and activity of digestive enzymes involved in food digestion [especially trypsin for digesting *A. salina* nauplii; see Bolasina et al. (2006)] differ widely between species (Chakrabarti et al., 1995; Hidalgo et al., 1999). Intraspecifically, this varies depending upon developmental stage (Baragi and Lovell, 1986; Baglole et al., 1998; Chakrabarti et al., 2006; Faulk et al., 2007), individual fitness (Bolasina et al., 2006), diet (Papoutsoglou and Lyndon, 2006), and

environmental factors. Depending upon fish species and type of digestive enzyme, particularly season and temperature may lead to gradually declining or even to full suppression of digestive enzymes' secretion and activity (Hidalgo et al., 1999; Kolkovski, 2001; Logothetis et al., 2001; Kofuji et al., 2005), including to decrease the digestive contribution of live food organisms themselves (Dabrowski and Glogowski, 1977a, b). In *M. fossilis* larvae incubated at 9 and 12 °C, ingested food particles often leave the digestive tract either only partially predigested (12 °C), or wholly undigested (9 °C). Moreover, ingestion of the first exogenous food even precedes the digestive system's activation (at 9 °C), which occurs simultaneously with the absolute yolk sac depletion (if it ever occurs).

The optimal temperatures for *M. fossilis* larvae survival lie in the range of 15–21 °C (24 °C is considered a suboptimal temperature, as survival rate 3 days post-onset of exogenous feeding declined appreciably), i.e. within a zone of lower temperatures in comparison to maximal growth (which spans from 21 to 24 °C, as determined by the fastest achievement of maximum size), as in striped bass *Morone saxatilis* (Walbaum) (Morgan et al., 1981), white perch *Morone americana* (Gmelin) (Morgan and Rasin, 1982) and rainbow trout *Oncorhynchus mykiss* (Walbaum) (Kato and Kamler, 1983).
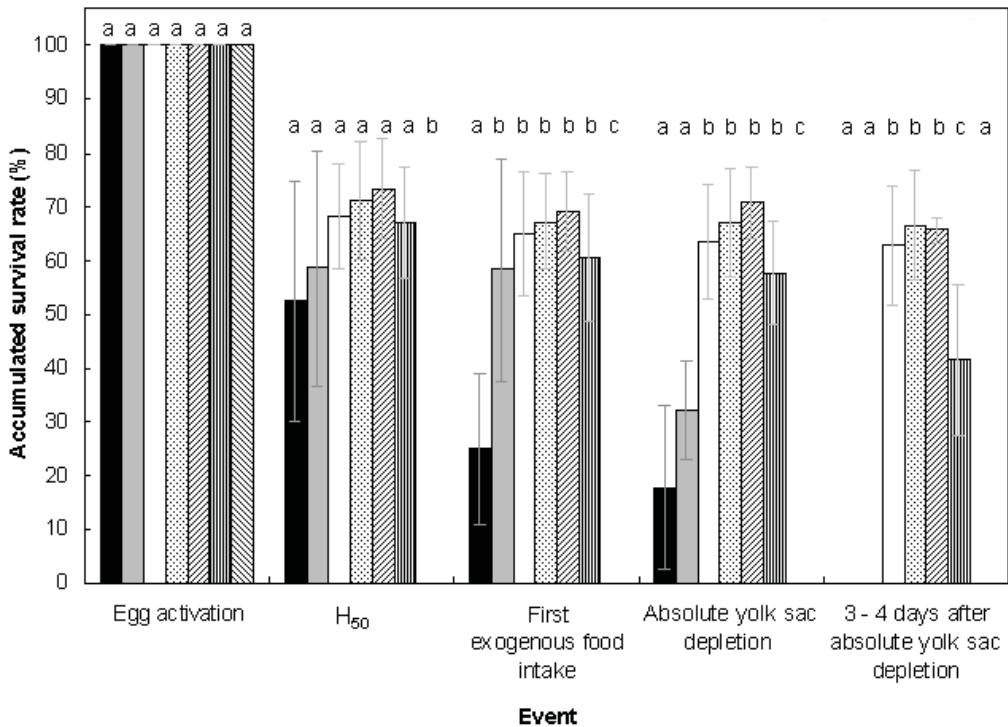


**Figure 14.** *Accumulated survival rate (mean ± S.D.; expressed in %) in relation to incubation temperature during early life history (from egg activation up to 3–4 days after absolute yolk sac depletion) in M. fossilis. Groups with the same superscript (a, b, c) do not differ significantly (Tukey HSD test, P = 0.05).*
*■, 9 °C; ▨, 12 °C; □, 15 °C; ▨, 18 °C; ▨, 21 °C; ▥, 24 °C; ▨, 27–36 °C;  $H_{50}$, point of hatching of 50% of individuals*

In summary, in *M. fossilis* the early life history (from egg activation to onset of exogenous feeding) is overall within a viable temperature range (9–24 °C and 15–24 °C for the embryonic and larval periods, respectively) a truly thermal-independent process in terms of yolk sac utilization efficiency [as in *C. nasus*; Kamler (1998)] and specimen outward appearances, as individuals achieve similar size and developmental stage at all key events regardless of temperature. By contrast, however, there is a time aspect in evaluating the onset of any crucial event (temporal prolongation at lower temperatures and vice versa), as in *C. nasus* (Kamler, 1998), and assessing any rate (survival, developmental and growth rate including temperature $Q_{10}$ coefficients).

In conclusion, *M. fossilis*, like the related species *C. taenia* (Bohlen, 2003), is a typical warm-mesothermic species (classification according to Wieser, 1991). Based on a combination of survival and energetic performance, 15–24 °C and 15–21 °C seem to be optimal temperatures for *M. fossilis* egg incubation and rearing of yolk-feeding larvae, respectively. Nevertheless, as shown in the present data, as well as by Kotlyarevskaja's (1967) observation of normally developing *M. fossilis* eggs at 7–7.5 °C in the wild (with oxygen concentration 3.2–9.2 ml.l$^{-1}$), *M. fossilis* is commonly able to tolerate such low temperatures as do coldwater fishes [e.g. most salmonids, esocids and percids, as summarized by Kamler (2002)] for relatively long periods as a response to periodic water temperature disturbances at the spawning localities during the spring mating season.

## ACKNOWLEDGEMENTS

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# CHAPTER 3

**PLOIDY LEVELS IN WEATHERFISH *Misgurnus fossilis* (L.)**

# BRIEF COMMUNICATIONS

## Sympatric occurrence of triploid, aneuploid and tetraploid weatherfish *Misgurnus fossilis* (Cypriniformes, Cobitidae)

B. Drozd*†, M. Flajšhans* and P. Ráb‡

*\*University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic and ‡Institute of Animal Physiology and Genetics, AS CR, v.v.i., Department of Vertebrate Evolutionary Biology and Genetics, Laboratory of Fish Genetics, 277 21 Liběchov, Czech Republic*

Ploidy analyses of 116 weatherfish *Misgurnus fossilis* individuals revealed the sympatric occurrence of triploid, intermediate aneuploid and tetraploid specimens in a 1:1:4 ratio. No diploids were detected and the sex ratio of triploids and tetraploids was 1:1, while that of aneuploids was skewed at 3:1 for males. An origin of intermediate aneuploids from mating triploids with tetraploids is hypothesized. © 2010 The Authors

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Key words: flow cytometry; genome size; loach; sex ratio; variation in natural polyploidy.

Available evidence about chromosome number and ploidy in weatherfish *Misgurnus fossilis* (L.) is inconsistent or contradictory. Raicu & Taisescu (1972), Boroń (2000) and Ene & Suciu (2000) report a diploid chromosome number of $2n = 100$ in specimens of Black Sea and Baltic Sea drainages. Vujošević *et al.* (1983), however, described individuals with diploid chromosome numbers of $2n = 50$ from the Moravica River (Danube Basin, Black Sea, Serbia). Finally, Palíková *et al.* (2007) in a genotoxicity study, used standard individuals with 100, but also with 50 chromosomes originating from farmed stock from the Morava River (Danube basin, Black Sea, Czech Republic). The *M. fossilis* individuals with 100 chromosomes undoubtedly behave as biological diploids (Boroń, 2000), but they very probably arose *via* an ancestral polyplodization event, as can be deduced from the suggestion of Ene & Suciu (2000) after a more detailed analysis of a karyotype consisting of chromosome quadruplets. To this intent, Palíková *et al.* (2007) arranged mitotic chromosomes of a specimen with 100 chromosomes into 25 quadruplets instead of 50 pairs. Diploid chromosome numbers of $2n = 50$ were reported in *Misgurnus* species *M. nikolskyi* Vasil'eva and *M. mohoity* (Dybowski), both by Vasil'ev & Vasil'eva (2008), while $2n = 48$ was shown in *M. mizolepis* Günter by Ueno *et al.*

†Author to whom correspondence should be addressed. Tel.: +420 383 382 402; email: drozd@vurh.jcu.cz

(1985) and Lee *et al.* (1987) and $2n = 48$ or 49 in large-scale loach *Paramisgurnus dabryanus* Dabry de Thiersant by Li *et al.* (1983) and Yu *et al.* (1989), respectively. A review of various forms of oriental weatherfish *Misgurnus anguillicaudatus* (Cantor) by Arai (2003), however, demonstrated the existence of diploids ($2n = 50$), triploids ($3n = 75$) and tetraploids ($4n = 100$ chromosomes) in the wild, as well as of pentaploids and hexaploids (125 and 150 chromosomes, respectively) produced by chromosome set manipulation.

The presence of *M. fossilis* specimens with different diploid chromosome numbers (100 or 50) points to the existence of at least two evolutionary lineages (with distinct ploidy levels), or even species separate from those presently recognized as *M. fossilis*, similar to the discovery of cryptic clonal lineages in *M. anguillicaudatus* by Morishima *et al.* (2002, 2008*a*).

Using data on geographical distribution of haplotype richness of misgurnids (Bohlen *et al.*, 2007; Mendel *et al.*, 2008) that survived over glacial maxima in the main refuge within the Danube basin and in putative additional refuges, ploidy levels in a natural misgurnid population inhabiting the Lužnice River floodplain area were studied.

Altogether, 116 specimens of *M. fossilis* were captured in the floodplain area pools on the upper reaches of the Lužnice River (tributary of the Vltava River, Elbe River basin, North Sea drainage) close to Majdalena (48° 97′ N; 14° 86′ E) in South Bohemia, Czech Republic, during 2007–2009. Localities were in the Třeboňsko National Protected Area. Each of the four pools within a 2 km range was completely electrofished three times per year. All specimens were transferred to the University of South Bohemia for investigations, individually marked with elastomer tags, and then released back into their original localities except for those sacrificed upon permit for head kidney chromosome preparation.

Before handling, fish were anaesthetized with $0.04$ ml $l^{-1}$ clove oil. Sex was determined from external markers of sexual dimorphism (Geldhauser, 1992). Blood was collected from the caudal vein into a heparinized syringe according to Pravda & Svobodová (2003), kept at $4°$ C and processed immediately. The ploidy level of each specimen was assessed by: (1) flow cytometry, (2) Feulgen image analysis densitometry, (3) erythrocyte nuclear dimensions and (4) direct karyotyping.

Flow cytometric histogram of $2n$, $3n$ and $4n$ specimens of *M. anguillicaudatus* was obtained from the Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Japan, as well as blood smears of these fish, which were then stained and measured in the authors' laboratory and used as standards.

Karyotyping of specimens analysed that were left alive was done by non-invasive method from cells of regenerating fin margins, according to the method of Völker & Ráb (2011), and from leukocyte culture derived from peripheral blood following the protocol by Fujiwara *et al.* (2001). Other specimens were karyotyped using direct chromosome preparation from the head kidney (Ráb *et al.*, 1987).

The ploidy level of each specimen was determined as the relative DNA content in erythrocytes using a Partec CCA I flow cytometer (Partec GmbH; www.partec.com) according to Linhart *et al.* (2006), first separately and then pooled. Erythrocytes of karyologically identified specimens with $2n = 100$ gave a relative DNA content of $4n$ as the tetraploid standard, which was used as internal control.

Blood smears were made according to Pravda & Svobodová (2003) and were stained with DNA Staining Kit according to Feulgen (Merck Co.; www.merck.com).

Feulgen image analysis densitometry followed the protocol of Hardie *et al.* (2002) to measure integrated optical density in erythrocyte nuclei and compute the *C*-value (haploid nuclear DNA content). Identically stained blood smears of chicken *Gallus gallus domesticus* and tench *Tinca tinca* (L.) were used as standards with *C*-values 1·25 and 1·00 pg DNA nucleus$^{-1}$, respectively (Gregory, 2009).

Feulgen-stained erythrocyte nuclei were measured for nuclear area (NA), nuclear major axes (NMA) and nuclear minor (NMI) axes, as described by Flajšhans (1997) and Svobodová *et al.* (1998). Nuclear volume ($V_{nucleus}$) was computed according to the formula for oblate spheroid or ellipsoid following Benfey & Sutterlin (1984).

Kolmogorov–Smirnov and non-parametric Kruskal–Wallis tests (including multiple comparisons of mean ranks for all groups tested in next step) in Statistica 7.0 (StatSoft, Inc.; www.statsoft.com) were used to check data normality and the effect of ploidy level on the respective variables. Values of $P = 0·01$ were considered significant.

Of 15 specimens karyotyped, 14 (seven females and seven males) possessed 100 chromosomes in somatic cells and therefore were considered to be tetraploids. These were used as tetraploid standards. A single female exhibited a modal number of 87 metaphase chromosomes (ranging from 79 to 94 per metaphase plate). No triploid karyotype was found among these 15 specimens.

Table I summarizes the results of the measured and computed variables for *M. fossilis* in comparison with *M. anguillicaudatus* standards.

The Kolmogorov–Smirnov test rejected data normality for all variables investigated.

Flow cytometry, Feulgen image analysis densitometry and erythrocyte nuclear dimensions revealed the presence of triploid, tetraploid and intermediate aneuploid specimens with significant differences among ploidy levels for all investigated variables, *i.e.* relative DNA content ($H_{2,115} = 91·23$, $P < 0·01$), *C*-value ($H_{2,115} = 40·00$, $P < 0·01$), NA ($H_{2,115} = 72·31$, $P < 0·01$), NMA ($H_{2,115} = 35·42$, $P < 0·01$), NMI ($H_{2,115} = 63·26$, $P < 0·01$) and $V_{nucleus}$ ($H_{2,115} = 71·28$, $P < 0·01$).

The erythrocytes of triploid, intermediate aneuploid and tetraploid individuals had average relative DNA contents equivalent to 3·19*n*, 3·53*n* and 4·00*n* (Fig. 1), respectively. The c.v. was at the same level for each ploidy group. Multiple comparisons of mean ranks for all groups revealed relative erythrocyte DNA content of triploid, intermediate aneuploid and tetraploid individuals to differ significantly from one another. Accordingly, *C*-values of erythrocytes from intermediate aneuploids and tetraploids differed significantly from that of triploids but not from one another. In contrast, all dimension variables (NA, NMA, NMI and $V_{nucleus}$) of triploids and intermediate aneuploids differed significantly from those of tetraploids but not from each other.

In total, 19 triploids, 20 intermediate aneuploids and 77 tetraploids were recognized in a 1:1:4 ratio regardless of sex. The sex ratio of triploids and tetraploids was 1:1 and differed from that of intermediate aneuploids (3:1 for males).

The *C*-value of erythrocytes from tetraploid individuals (2·02 ± 0·09 pg nucleus$^{-1}$) in this study was a slightly lower value when compared to the database record for *M. fossilis* specimens with 100 chromosomes [2·60 pg nucleus$^{-1}$; Timofeeva & Kaviani (1964) in Gregory (2009)] which was based upon a different analytical method.

A bisexually reproducing fish population with regular gametogenesis cycles behaves as a biological diploid regardless of its chromosome number (Ráb &

TABLE I. Ploidy level data of *Misgurnus fossilis* (mean ± s.d.): relative DNA content (channel number and coefficient of variation, c.v.), $C$-value, erythrocyte nuclear dimensions [nuclear area (NA), nuclear major axis (NMA), nuclear minor axis (NMI) and nuclear volume ($V_{nucleus}$)]. Data of *Misgurnus anguillicaudatus* standards are given below with the same superscript lower case letters do not significantly differ at $P < 0.01$. Groups

| Ploidy level | Relative DNA content | | | $C$-value (pg DNA nucleus$^{-1}$) | NA (μm$^2$) | NMA (μm) | NMI (μm) | $V_{nucleus}$ (μm$^3$) |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Channel number | c.v. (%) | Ploidy level | | | | | |
| *M. fossilis* | | | | | | | | |
| Triploid (3n) | 79.78 ± 1.55[a] | 2.13 ± 0.85 | (3.19n) 3n | 1.70 ± 0.10[a] | 14.61 ± 1.44[a] | 5.25 ± 0.39[a] | 3.36 ± 0.20[a] | 31.19 ± 4.44[a] |
| Aneuploid (aneu) | 88.33 ± 2.16[b] | 1.82 ± 0.51 | (3.53n) 3/4n | 1.98 ± 0.19[b] | 15.46 ± 0.67[a] | 5.58 ± 0.29[a] | 3.34 ± 0.12[a] | 32.56 ± 1.89[a] |
| Tetraploid (4n) | 100.98 ± 4.31[c] | 2.02 ± 0.83 | (4.00n) 4n | 2.02 ± 0.09[b] | 18.41 ± 1.66[b] | 5.87 ± 0.33[b] | 3.77 ± 0.17[b] | 43.53 ± 4.12[b] |
| *M. anguillicaudatus* standards | | | | | | | | |
| Diploid (2n) | 102·42 | 2.20 | (2.04n) 2n | 1.65 ± 0.05 | 15.94 ± 0.68 | 6.49 ± 0.09 | 2.81 ± 0.11 | 26.82 ± 1.98 |
| Triploid (3n) | 155·16 | 2.09 | (3.08n) 3n | 2.54 ± 0.04 | 19.94 ± 1.20 | 7.98 ± 0.96 | 3.01 ± 0.03 | 37.84 ± 2.74 |
| Tetraploid (4n) | 201·29 | 2.11 | (4.00n) 4n | 3.16 ± 0.20 | 24.23 ± 1.96 | 8.56 ± 0.60 | 3.29 ± 0.59 | 48.49 ± 2.17 |
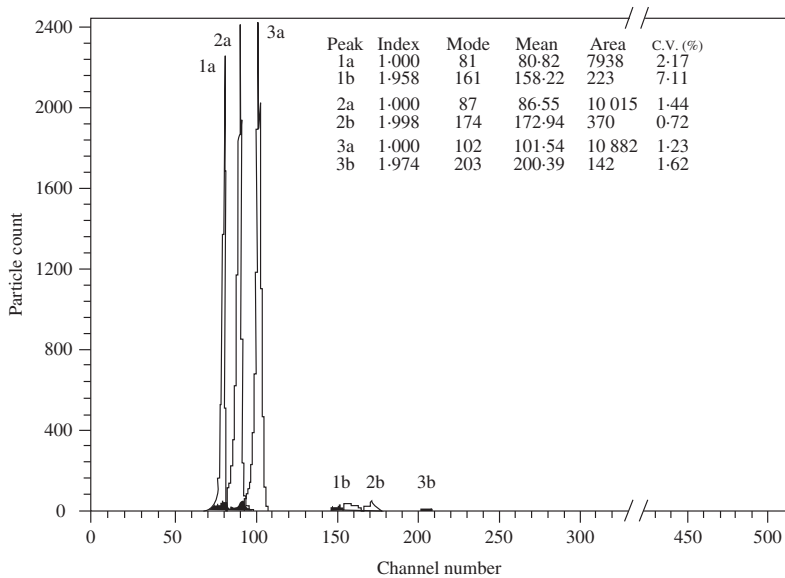
FIG. 1. Relative DNA content in *Misgurnus fossilis*: (1a) erythrocytes of a triploid ($3n$) specimen, (2a) erythrocytes of an intermediate $3n-4n$ aneuploid specimen, (3a) erythrocytes of a tetraploid ($4n$) specimen. Small peaks (1b, 2b and 3b) show erythrocyte doublets in the respective specimens.

Collares-Pereira, 2004). Nevertheless, sporadic data in the literature consider *M. fossilis* with $2n = 100$ or $2n = 50$ chromosomes. To distinguish such situations, specimens with 50 chromosomes are named as diploids and specimens with 100 chromosomes as tetraploids. No report on *M. fossilis* ever mentioned sympatry of such tetraploid and diploid individuals within one natural locality.

At the localities under study, however, not only a sympatric occurrence of tetraploid and triploid individualsss but also the existence of intermediate aneuploid individuals, *i.e.* ploidy levels also recognized by Arai *et al.* (1991) and Zhang & Arai (1999*a*, *b*, 2003) in another species, *M. anguillicaudatus* were found.

The frequency of particular ploidy levels in combination with their sex ratios suggested a prevailing majority of tetraploids (66% of specimens studied) in equal proportion for both sexes. Nevertheless, triploids and intermediate aneuploids were also notably represented within the wild *M. fossilis* population studied (*c.* 17% of specimens for each), and the two groups also differed in their sex ratios.

A possible origin of triploids could be the addition of a sperm genome to an unreduced diploid egg, as in the case of *M. anguillicaudatus* (Zhang *et al.*, 1998; Itono *et al.*, 2007; Morishima *et al.*, 2008*a*) or in the *Cobitis* species complex (Vasil'ev *et al.*, 1989; Janko *et al.*, 2007). In addition, hypothetical crossing of *M. fossilis* forms with $2n = 50$ and $2n = 100$ chromosomes cannot be excluded. The survival and spread of triploids in a natural population of *M. fossilis* remains unknown, because fertility of triploid *M. fossilis* has not yet been investigated. The balanced sex ratio 1:1 in triploids, however, might suggest their fertility and usual bisexual reproduction as described, *e.g.* for batura toad *Bufo baturae* (Stöck *et al.*, 2002).

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In *M. anguillicaudatus*, however, Zhang *et al.* (1998) found only sterile triploid males and Oshima *et al.* (2005) found triploid males mostly producing unreduced sperm incapable of fertilization, and only a negligible concentration of haploid sperm. On the other hand, natural clone-derived *M. anguillicaudatus* triploids form mainly 1*n* eggs, sometimes 2*n*, 3*n* and aneuploid eggs (Oshima *et al.*, 2005; Morishima *et al.*, 2008*b*). Another type of wild *M. anguillicaudatus* triploid (originating from tetraploid × diploid mating) produced 1*n* and unreduced 3*n* eggs simultaneously (Zhang *et al.*, 1998).

Alternatively, asexual gynogenetic reproduction of triploids allowing survival and independent spreading in a bisexually reproducing lineage could explain this phenomenon, as in the case of spined loaches *Cobitis* sp. (Janko *et al.*, 2007).

Intermediate aneuploids in the present study showed true intermediate character between triploid and tetraploid, demonstrated by chromosome number and relative DNA content (Fig. 1), and were thus probably unaffected by chromosome losses during preparation. With Feulgen image analysis densitometry, however, intermediate aneuploids were often hidden within tetraploids. With erythrocyte dimension analysis, they were often hidden within triploids. According to Zhang & Arai (1999*b*), such individuals, described as hyper-triploids, probably arose from fertilization of a diploid egg of a tetraploid female by an aneuploid sperm of an artificially induced triploid male. The present results, like the conditions discovered by Arai & Inamori (1999) and Zhang & Arai (1999*a, b*) in *M. anguillicaudatus*, thus suggest a certain tolerance of *M. fossilis* to polyploidy as well as to aneuploidy. Further karyotype studies are therefore highly desirable. Findings of intermediate aneuploids, in the present study, might indicate hypothetical fertilization capacity of natural triploid individuals in *M. fossilis*, as well as possible hybridization of sympatrically living triploid and tetraploid individuals.

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# CHAPTER 4

GENERAL DISCUSSION ◆ ENGLISH SUMMARY ◆ CZECH SUMMARY ◆ ACKNOWLEDGEMENTS ◆ LIST OF PUBLICATIONS ◆ TRAINING AND SUPERVISION PLAN DURING STUDY ◆ CURRICULUM VITAE

# GENERAL DISCUSSION

The floodplain river areas gradually lost their ecological function in providing suitable fish spawning and nursery areas during the second half of the 20th century, as a response to water quality degradation and regulation of the entire river systems (Bain et al., 1988; Ward and Stanford, 1995; Aarts et al., 2004). These man-made activities led to general rapid decrease in fish species richness and decline in abundance of particular riverine species, especially rheophilic fishes (*B. barbus*, *C. nasus*) (Mann, 1996), which consequently became a very important indicator group contributing to estimation of river integrity (Karr, 1991). As it is true in rheophilic species, the *M. fossilis* is among those fishes crucial for explaining the importance of river inundation areas in respect to successful fish reproduction, as well as to distribution and dynamics of early developmental stages (Ward, 1998) that present the key events for fish recruitment and population dynamics (Kamler, 1992; Schiemer et al., 2003). As a consequence of the preceding, the principal goals of the present Ph.D. thesis were in experimental evaluation of thermal sensitivity of early life history, as well as in elucidation of the question which ploidy levels occur in natural *M. fossilis* population inhabiting the floodplain area of the Lužnice River, in order to predict wild population dynamics and life demands of *M. fossilis* for purposes of effective conservation management.

## TEMPERATURE-INDUCED PLASTICITY OF EARLY LIFE HISTORY IN WEATHERFISH *Misgurnus fossilis* (L.)

### Female fecundity parameters

The first fecundity parameter of weatherfish *Misgurnus fossilis* (L.) females, i.e. the absolute stripping fecundity observed by Drozd et al. (2009; ca $6.90.10^3$ eggs per female on average) represented a similar value to that showed in study of Bohl (1993), but ca 20%, 50% or even 60% lower than data shown by Geldhauser (1992), Kouril et al. (1996) or Adamkova-Stibranyiova et al. (1999), respectively. A comparable situation to the previous magnitude was recorded by Drozd et al. (2009) in case of both other parameters, i.e. the relative stripping fecundity ($121.60.10^3$ eggs.kg$^{-1}$ b. m. on average) and the relative mass of stripped eggs (10.70% of the original female body mass prior to stripping on average). These data were in average about one half of the values of Geldhauser (1992), Kouril et al. (1996) and Adamkova-Stibranyiova et al. (1999). The reason for this discrepancy might be found in origin of the broodstock. Drozd et al. (2009) used adults from the nature (fish spent in captivity only 14 days prior to the stripping). The other above-cited authors used broodstock that has been reared in intensive or extensive pond culture over the whole season, i.e. in environment with relative sufficiency of food resources and stable water conditions in terms of temperature and chemistry. In fact, Drozd et al. (2009) could obtain just only one portion of eggs. The *M. fossilis* belongs to species inhabiting the environment with fluctuating conditions often tending to unfavourable levels (high ammonium content and high sulphate concetration) and frequent temperature-, pH- and water level disturbances (Meyer and Hinrichs, 2000; Pekárik et al., 2008). Such fish often exhibit multiple- or fractional spawning strategy in order to amplify their chance for successful reproduction and survival at the natural localities of occurrence, as described by Bohlen (1999b) for the related spined loach *Cobitis taenia* (sensu L.) upon laboratory experiments. To date, however, there are no data of that kind for *M. fossilis*.

As it was summarized by Kamler (1992, 2005) in general, all the above described female fecundity parameters should be for that reason considered only the approximate magnitudes in *M. fossilis* (because of inaccuracies), highly dependent on other biological variables of the fish, such as age,

size, health conditions, natural and artificial influences during the pre-spawning period (temperature, application of hormonal preparations and their dose) and the spawning period (including stripping experience). However, in endangered and protected fish species such as *M. fossilis*, this method is the only one possible way to estimate the necessary data without any need to kill the adult fish (Drozd et al., 2009).

Both the average wet egg mass (0.88 mg on average) and egg diameter (1.42 mm on average) observed by Drozd et al. (2009) were significantly lower compared to the figures showed by Kouril et al. (1996), Adamkova-Stibranyiova et al. (1999) for wet egg mass (decrease ca 10–15%); and by Kryzanovskij (1949), Kostomarova (1975) for egg diameter (decrease ca 13–23%), respectively. In an interspecific comparison, *M. fossilis* has bigger eggs (in terms of egg diameter, e. d.) compared to *M. anguillicaudatus* [e. d. = 0.72–0.84 mm; Zheng (1985)], mud loach *Misgurnus mizolepis* (Günther) [e.d. = 1.10 mm; Kim et al. (1987)], *C. taenia* [e.d. = 1.14 ± 0.07 mm; Bohlen (1998)] and *Cobitis bilineata* Canestrini [e. d. = 1.21 ± 0.09 mm; Bohlen (1998)], respectively.

Nevertheless, we have to bear in mind a fact that the average wet egg mass and egg diameter seem to be considerably influenced by yolk mass volume and are dependent on the other female reproductive traits (Pepin et al., 1997; Marteinsdottir and Steinarsson, 1998) which vary interseasonally, as well as during one individual season, and intraspecifically i.e. among populations (Kamler, 1992; Marteinsdottir and Steinarsson, 1998) too. That is to say, a serious comparison or deep analysis in this respect is very difficult to make due to lack of data regarding the female condition (especially age, size, spawning experience and fecundity) in available evidence (Drozd et al., 2009).

## Development

Temperatures within the presumed optimum range (15–24 and 15–21 °C for embryonic and larval periods, respectively; see below) accelerate the development of *M. fossilis* in terms of: (a) incubation and (b) hatching periods duration [amplitudes of both magnitudes at minimal temperature reach approximately nine times and fifteen times, respectively, the value for the maximal temperature – Drozd et al. (2009)], as well as (c) time of Ac, S, Re observations, during different intervals of the embryonic and larval periods in a uniform (inversely proportional) way (Drozd et al., 2010a), as is the case for common carp *Cyprinus carpio* L. (Penaz et al., 1983), vendace *Coregonus albula* (L.) (Luczyński and Kirklewska, 1984) and sea trout *Salmo trutta trutta* L. (Raciborski, 1987).

Values of the incubation period amplitude (in range 18–24 °C) shown by Drozd et al. (2009) represent a comparable level presented by Kostomarova (1975) (at 21.50 °C), Kouril et al. (1996) (at 18 °C) in *M. fossilis*; and by Watanabe et al. (1948) (20–21 °C), Suzuki (1953) (19–21 °C) and Fujimoto et al. (2006) (20 °C) in related species *M. anguillicaudatus*, respectively.

However, Grieb (1937), Geldhauser (1992) and Kryzanovskij (1949) in *M. fossilis*, and Zheng (1985) in *M. anguillicaudatus*, offer noticeably different figures compared to the preceding ones, which probably originated either in an unknown origin of the incubated eggs [Grieb (1937) used fertilized eggs with unknown age from the nature, which were subsequently incubated at temperature 15 °C in laboratory] or in the unstable water conditions during egg incubation (temperature varied in range 16–20 °C, 19.50–23 °C and 15–16.90 °C in case of laboratory experiments made by Kryzanovskij (1949), Zheng (1985) and Geldhauser (1992), respectively). Data of duration of the incubation period presented by them, however, correspond to situation of egg incubation under different water conditions (than which were originally mentioned), i.e. at constant temperature 12 °C, at fluctuating temperature varying in range 15–18 °C, around 24 °C and in range 15–21 °C, respectively – comparison made according to results presented by Drozd et al. (2009, 2010a) (see Figure 4 in Chapter 2.1.).

In *M. fossilis* embryos, the threshold temperature ($t_0$) and effective day-degrees ($D°_{eff}$) are 8.36 °C and 28.74, respectively (Drozd et al., 2010a). Thus, within Kamler's (2002) classification of temperature requirements for embryonic development considering the relationship between $t_0$ and $D°_{eff}$, *M. fossilis* occupies an intermediate, ambivalent position near that of phytophilous cyprinids spawning like weatherfish in stagnant waters during late spring and early summer (Kottelat and Freyhof, 2007), i.e.: (1) in $t_0$ terms, comparable to lake minnow *Eupallasella percnurus* (Pallas) ($t_0$ = 8.30 °C, $D°_{eff}$ = 45.50; Kamiński et al., 2006) and bream *Abramis brama* (L.) ($t_0$ = 8.60 °C, $D°_{eff}$ = 41.00; Kamler, 2002), and (2) in $D°_{eff}$ terms, *C. carpio* ($t_0$ = 9.90 or 11.10 °C, $D°_{eff}$ = 30.00 or 34.00; Kamler, 2002), tench *Tinca tinca* (L.) ($t_0$ = 11.70 °C, $D°_{eff}$ = 29.00; Kamler, 2002) or the nearby lithophilous species, vimba *Vimba vimba* (L.) ($t_0$ = 10.10 °C, $D°_{eff}$ = 28.00; Kamler, 2002).

Available evidence as to the effect of temperature upon hatching size in ray-finned fishes is inconsistent and contradictory. Larval size at hatch commonly is inversely related to temperature (lower temperatures produce bigger larvae), i.e. hatching is generally considered an age-related rather than size-related event (Penaz et al., 1983; Kamler et al., 1994, 1998; Ojanguren and Braña, 2003; Martell et al., 2005). Contrary to the preceding, Alderdice and Forrester (1974) and Pepin et al. (1997), respectively, reported on a significant decline in size of freshly hatched flathead sole *Hippoglossoides elassodon* Jordan and Gilbert and Atlantic cod *Gadus morhua* L. larvae with decreasing temperature (in the latter, an effect of increased metabolic requirements at temperatures close to the lower boundary of thermal tolerance range is supposed). However, as in Atlantic herring *Clupea harengus* L. (Blaxter and Hempel, 1963), Black Sea salmon *Salmo labrax* Pallas (Zalicheva, 1981), haddock *Melanogrammus aeglefinus* (L.) (at 2–8 °C, Martell et al., 2005) and *G. morhua* (only batch 2, Jordaan et al., 2006), size of newly hatched *M. fossilis* larvae (Drozd et al., 2009, 2010a) is neither positively nor negatively correlated with temperature ($L_T$ of larvae at hatch does not significantly differ based upon temperature except at 12 °C; see comments in Results). In addition, it may be supposed that (1) length thus might determine the age at hatching, rather than the age at hatching is determining the hatching length in *M. fossilis*; and (2) growth within eggs is most likely unequal, and thus a synergistic effect of both embryonic growth and temperature probably determines the hatching time in *M. fossilis* [at least within the temperature range studied – Drozd et al. (2009, 2010a)].

A size of newly hatched larvae seems to be considerably influenced by parental attributes (Panagiotaki and Geffen, 1992; Wootton, 1990; Kamler, 2005) and water conditions (Keckeis et al., 1996; Prokes et al., 1998; Schiemer et al., 2003). Drozd et al. (2009, 2010a) observed a significant decline in total length of freshly hatched *M. fossilis* larvae (decrease ca 15% on average) compared to the figures presented by Grieb (1937), Kryzanovskij (1949), Kotlyarevskaja (1967) and Kostomarova (1975), what was probably derived from the smaller egg size used by Drozd et al. (2009, 2010a). According to Kotlyarevskaja (1967), a ground of this variance might be found also in oxygen level (total length of freshly hatched *M. fossilis* larvae reached ca 5 mm and ca 4 mm in treatment with oxygen concentration 6.00–8.50 $mgO_2.l^{-1}$ and 2.40–4.00 $mgO_2.l^{-1}$, respectively), but Drozd et al. (2009, 2010a) measured oxygen concentration overreaching 7 $mgO_2.l^{-1}$ on average (ca 90% of oxygen saturation) over all temperatures. Compared to other loach species, nevertheless, size of *M. fossilis* larvae after hatching (Drozd et al. 2009, 2010a) commonly reaches twice the value for related species *M. anguillicaudatus* recorded by Zheng (1985) (ca 1.95–2.40 mm) and Fujimoto et al. (2006) (ca 2.60 mm on average).

Developmental stage of freshly hatched larvae is commonly negatively related to incubation temperature (Penaz et al., 1983; Ojanguren and Braña, 2003), i.e. the hatching event is only loosely linked to a specific developmental stage in fishes (Yamagami, 1988). Nevertheless, developmental stage of newly hatched *M. fossilis* larvae (Drozd et al., 2009, 2010a) shows only a slight declining tendency upon rising temperature [stage 37 at 9–18 °C and stage 36 at 21–24 °C; Kostomarova's (1975)]. This may not be explained, however, through other water condition (especially oxygen concentration),

as in Kotlyarevskaja (1967) [because of oxygen saturation approximately 90% for each temperature treatment in the studies of Drozd et al. (2009, 2010a) – see above].

According to the criteria of Kamler (2002), no apparent influence of incubation temperature upon either the size or developmental stage of newly hatched *M. fossilis* larvae (Drozd et al., 2009, 2010a) may be evidence of a very wide zone of viable temperatures for *M. fossilis* – at least during the embryonic period (from Fe to H).

The size of *M. fossilis* larvae at the onset of mixed (S), as well as exogenous (Re), feeding was found by Drozd et al. (2010a) to be independent of temperature (except at 9 °C), as in the cases of yellowtail flounder *Limanda ferruginea* (Storer) (Howell, 1980), summer flounder *Paralichthys dentatus* (L.) (Johns et al., 1981), turbot *Psetta maxima* (L.) (Quantz, 1985), *Chondrostoma nasus* (L.) (Kamler et al., 1998), *C. harengus* (Geffen, 2002), and *M. aeglefinus* (Martell et al., 2005). The developmental stage of larvae at Re can be considered temperature independent in *M. fossilis* as well  [all larvae show traits associated with two of Kryzanovskij's (1949) late larval stages, i.e. stages at the ages of 12 and 26 dPF] (Drozd et al., 2010a).

As already stated above, *M. fossilis* inhabits waters often suffering temperature, pH and water level disturbances, as well as high ammonium and sulphate but low oxygen concentrations (Meyer and Hinrichs, 2000; Pekárik et al., 2008). That is to say, their localities generally represent unfavourable and ever-changing environments. Weatherfish larvae hatch from sticky eggs attached to underwater vegetation and hang fast to plants by the head for a couple of days (Grieb, 1937; Kryzanovskij, 1949; Kostomarova, 1975). Consequently, the similar size and developmental stage of *M. fossilis* larvae at every key event – i.e. at hatching (H$_{50}$), as well as at the onset of mixed (S) and exogenous (Re, yolk sac full depleted) feeding – regardless of temperature may be perceived as either a functional compromise between ontogenetic and behavioural traits conditioned by both endogenous and exogenous (abiotic and biotic) factors or an outcome from a set of various anatomical, physiological and developmental adaptations conducive to successful reproduction and survival in such environmental conditions (Drozd et al., 2010a).

Upon hatching, and for somewhat different reasons, both immature larvae [at higher temperatures, i.e. at 21–24 °C in study of Drozd et al. (2009, 2010a)] and overly mature larvae [at lower temperatures, i.e. 9–12 °C in study of Drozd et al. (2009, 2010a)] must not leave the eggs. Immature, immotile individuals face a risk of early death after hatching into life-inhibiting conditions, as the essential, additional respiratory organs will develop later [the first one, the outer filamentous gills, develops at Kostomarova's (1975) stage 38]. Also at risk are the over-mature individuals close to full yolk sac depletion, which have low energy resources stored in the yolk sac as a result of their prolonged time spent in the egg at lower temperatures. After hatching, they can face starvation leading quickly to death [according to Kamler (1992)].

From the viewpoint of predetermined larval size at hatching, the biotic interactions – in particular interspecific food competition and predation (Wootton, 1990; Kamler, 2002) – may be as significant as are the abiotic environmental conditions (Keckeis et al., 1996; Prokes et al., 1998; Schiemer et al., 2003) and parental attributes (Panagiotaki and Geffen, 1992; Kamler, 2008). This is because these conditions and attributes determine initial food size during the transition to both mixed and exogenous feeding (larger, better developed larvae have a greater chance to succeed in the interspecific food competition), and these further determine the size of predator able to utilize the particular developmental stages (immature larvae are more vulnerable to predation) (Wootton, 1990). Prospects for greater survival rates in larvae might be improved by rapid growth and by variation in behaviour (Eeton and DiDomenico, 1986; Webb and Weihs, 1986) associated with continually increasing maximum and mean escape speed (Green and Fisher, 2004) during the first couple days after hatching. Moreover, it looks like in *M. fossilis* "something specific and new" is required for transition to exogenous nutrition and thus to colonization

and utilization of new microhabitats and food resources. [Kotlyarevskaja (1967) observed weatherfish larvae actively climbing over aquatic vegetation in search of suitable food.] In context of preceding, Drozd et al. (2010a) observed *M. fossilis* larvae, which at the onset of exogenous feeding achieved that of Kryzanovskij's (1949) stages where they possess rudimentary outer filamentous gills, but also well-developed segmental blood vessels in the dorsal and anal parts of the fin-fold, as well as in pectoral fins, and considerably enlarged peri-intestinal blood vessels. Consequently, and regardless of temperature, *M. fossilis* larvae thus do not fully deplete the yolk mass, i.e. they do not start to utilize the exogenous nutrition only, until the gut becomes one of the functional additional respiratory organs (Drozd et al., 2010a).

Drozd et al. (2010a) revealed that the two-stage curve (Kramer and Zweifel, 1970) fitted by polynomial function might provide a useful model for both growth (expressed as $L_T$ increase in time) as well as for the course in time of the standard, pre-anal, post-anal, pre-orbital and orbital lengths for *M. fossilis* larvae reared over a wide temperature range. At 9 °C, however, this rule is violated (see increased metabolic requirements below). In *M. fossilis*, as in fishes generally (Kamler, 1992, 2002), a period of moderate increase in size shortly after hatching is followed by a phase of minimal growth and then by a period of rapid growth that comes soon after the onset of mixed feeding (S) [corresponding to a notable inflection region in the course of all morphometric characteristics studied by Drozd et al. (2010a)]. In orbital length, however, a course inflection point is moved backwards, i.e. the eyes as one of the sensory organs accelerate their development and start rapidly to grow relatively long before the onset of mixed feeding itself (Drozd et al., 2010a). This probably is a response to environmental needs, and especially timely food detection (Ibrahim et al., 2006; Mukai et al., 2008) and predator avoidance (Fuiman, 1994; Poling and Fuiman, 1997).

The duration of yolk mass depletion typically declines with rising temperature within the entire viable temperature range (summarized by Kamler, 1992, 2008). However, no effect of increasing water saturation within body tissues (including the yolk mass as well) by decreasing temperatures during early ontogeny, as supposed e.g. by Kamiński et al. (2006) in *E. percnurus*, is confirmed by Drozd et al. (2010a) in *M. fossilis* over the larval period (as there was no statistical difference in yolk sac volume among temperatures except for at 12 °C).

In an interfamilial comparison, cobitids – *M. fossilis* [ca 4–6% of the original yolk reserves at hatching (denotes as Y.r.) expressed as the yolk sac volume at $H_{50}$; Drozd et al. (2010a)] and flatfishes – *P. maxima* (ca 8% of Y.r. – Quantz, 1985) have available very low energy reserves deposited in the yolk sac at the onset of mixed feeding (S) compared to salmonids – Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (ca 20% of Y.r. – Heming, 1982) and *S. trutta trutta* (ca 55% of Y.r. – Raciborski, 1987) or centrarchids – largemouth bass *Micropterus salmoides* (Lacepède) (ca 21% of Y.r. – Laurence, 1969). In addition, Ito and Suzuki (1977) observed in the related species *M. anguillicaudatus* that larvae fed on detritus only and ingested no planktonic organisms. From an evolutionary viewpoint, both of these facts might support a presumption of relatively low dietary specialization and demands of loach larvae (according with a certain guarantee of finding sufficient suitable food regardless of abiotic factors actually existing at the locality of occurrence) (Drozd et al., 2010a). To date, however, nothing is known about the actual dietary requirements or foraging behaviour of *M. fossilis* during early life history.

Drozd et al. (2010a) revealed, that *M. fossilis* converted during the endogenous feeding period from hatching to the onset of mixed feeding (H–S), more of its yolk mass to body tissue (as expressed by the ratio of total length achieved and undigested yolk sac reserves) as incubation temperature was decreasing. This is comparable to tautog *Tautoga onitis* (L.) (Laurence, 1973), Atlantic salmon *Salmo salar* L. (Hamor and Garside, 1977) and *O. tshawytscha* (Rombough, 1985). In addition, according to Kokurewicz (1969), Alderdice et al. (1988) and Kamler (1992), an optimum temperature in terms of yolk mass efficiency occurred in *M. fossilis* at the intermediate (12–15 °C), i.e. not at the highest,

viable temperatures (Drozd et al., 2010a). At temperatures above and below this range, the metabolic requirements are commonly proportionally greater than those contributing to tissue production. This results in retarded growth (Johns et al., 1981; Blaxter, 1992; Kamler, 1992; Pepin et al., 1997) and thus in noticeably smaller larvae. On this basis, and combined with a low survival rate, 9 °C should be considered a temperature lying already close to the lower boundary of the thermal tolerance range for the early larval stages of *M. fossilis* (Drozd et al., 2010a).

In general terms, in *M. fossilis* yolk mass conversion efficiency from egg activation to absolute yolk sac depletion (expressed in terms of final larval total length) increases with rising temperature within the low temperature range (9–12 °C), but within the circum-(sub)optimal temperature range (12–24 °C) no apparent effect of temperature upon yolk sac utilization efficiency was observed by Drozd et al. (2010a). This is similar to observations regarding *C. harengus* (range: 3.5–17 °C; Overnell, 1997) and *C. nasus* (range: 10–19 °C; Kamler et al., 1998).

A general pattern of $Q_{10}$ declining with rising temperature over the entire early life history (summarized by Kamler, 2002) is highlighted in the *M. fossilis* embryonic period by a markedly elevated $Q_{10\,dev}$ value within the 9–12 °C range (triple the $Q_{10\,dev}$ value for the adjacent interval of higher temperature), as well as by an apparent violation of this common pattern observed for developmental rate within the range 15–18 °C ($Q_{10\,dev}$ reached twice the value for the adjacent interval of lower temperature) (Drozd et al., 2010a). Both of these facts may be useful in determining presumable temperature requirements for *M. fossilis* embryonic development. The former probably evidences a shift from a zone of temperatures close to lethality to one of suboptimal temperatures and the latter to an onset of optimal temperatures, which is also supported by limitation upon the rate of developmental progress ($Q_{10\,dev} = 1$) in the interval 21–24 °C (Drozd et al., 2010a).

Based on comparison of $Q_{10}$ values for developmental rate ($Q_{10\,dev}$) and growth rate ($Q_{10\,gr}$), it might be assumed that the ontogeny and growth of *M. fossilis* (Drozd et al., 2010a), like those of African catfish *Clarias gariepinus* (Burchell) (Kamler et al., 1994) and *C. nasus* (Kamler et al., 1998), are accelerated by temperature in a similar way (including the comparable values for both coefficients in the range of 15–24 °C in *M. fossilis*) over the entire period of larval endogenous feeding. Only the extents of $Q_{10\,dev}$ and $Q_{10\,gr}$ acceleration within temperature ranges 9–12 and 12–15 °C differ from one another, thus suggesting a stronger enhancement of growth compared to development during transition to the temperature conditions suboptimal and optimal for larval development in *M. fossilis* (Drozd et al., 2010a).

## Survival

Based on data shown by Drozd et al. (2009, 2010a) on *M. fossilis* eggs and larvae incubated at stable temperatures through the entire early ontogeny, which have recorded remarkably different effects of temperature on the particular stages of early ontogeny (especially in terms of survival), it is highly recommended by them to distinguish and separately evaluate the impacts of a wide temperature range on the embryonic and larval periods of weatherfish.

In *M. fossilis* (according to results shown by Drozd et al., 2009, 2010a), the zone of tolerated temperatures for the embryonic period (Fe–H) lies between ca 9 °C [development is theoretically arrested at ca 8.50 °C; the lowest viable temperature is mathematically estimated at ca 9.10 °C; Drozd et al. (2009, 2010a)] and ca 24 °C. An assumed upper lethal temperature lies in the 24–27 °C range, when survival rapidly declines from an above-average value (ca 70%) to 0%. Temperatures above 24 °C are thus by Drozd et al. (2009, 2010a) believed to be lethal due to a complete life-inhibiting effect already during the embryonic period. A thermal optimum [i.e. survival over 60% according to criteria of

Kostomarova (1975), Penaz et al. (1983) and Ozernyuk et al. (1987)] for *M. fossilis* embryonic development suggested by the observations of Drozd et al. (2009, 2010a) (range of 15–24 °C) corresponds to thermal conditions similar to those observed in nature by both Grieb (1937) and Kryzanovskij (1949) during the spawning season of *M. fossilis*, as well as the optimal temperatures proposed by Kostomarova (1975) [(13)14–(20)24 °C], Alexeeva and Ozernyuk (1987) or Zdanovich et al. (2001) (14–22 °C) based on experiments in laboratory conditions. Temperatures fewer than 15 °C should be considered suboptimal for the embryonic period [survival at 9 and 12 °C exceeded 50%, Drozd et al. (2009, 2010a)]. In an interspecific comparison, the zone of tolerated temperatures for the embryonic period in *M. fossilis* (Drozd et al., 2009, 2010) is shifted towards the lower temperatures compared to that in related loach species *M. anguillicaudatus*, in which Watanabe et al. (1948) observed normally proceeding hatching at 28–30 °C, and for which Kubota and Matsui (1955) and Wang et al. (2008), respectively, described the thermal optimum to be 25 °C and 25–27 °C. Nevertheless, 28 °C might be considered a critical or border-line temperature inducing sperm incorporation in *M. anguillicaudatus* (Itono et al., 2007).

In *M. fossilis*, however, based on data shown by Drozd et al. (2010a), the larval period is characterized by continuous and even more enhanced influence of temperature on development in terms of survival as an effect of stable thermal conditions over the entire period of early life history (especially at lower temperatures), which is reflected in a narrower viable temperature range (15–24 °C) than could be expected based upon a theoretical assumption for the larval data set [development should be theoretically arrested at ca 7.00 °C; the lowest viable temperature is mathematically estimated at ca 7.80 °C; Drozd et al. (2010a)] and the common pattern of thermal sensitivity of particular stages in early life history (larvae should tolerate a wider temperature range than do embryos, as summarized by Kamler, 2002).

Negative effects of lower temperatures (9 °C and 12 °C) on *M. fossilis* larvae development in terms of survival, apparent prolongation of yolk sac depletion, and reduced developmental and growth rates (especially at 9 °C) were observed by Drozd et al. (2010a) during the entire post-hatching period. A real lethal impact of these temperatures is not expressed, however, until the onset of exogenous feeding (i.e. until absolute yolk sac depletion), when all *M. fossilis* individuals will die within the next few days (Drozd et al., 2010a). This might probably be attributed to poor food intake ability or insufficient ability to digest ingested food particles at lower temperatures leading up to complete energy exhaustion. Generally, types, secretion and activity of digestive enzymes involved in food digestion [especially trypsin for digesting brine shrimp *Artemia salina* (L.) nauplii; see Bolasina et al. (2006)] differ widely between species (Chakrabarti et al., 1995; Hidalgo et al., 1999). Intraspecifically, this varies depending upon developmental stage (Baragi and Lovell, 1986; Baglole et al., 1998; Chakrabarti et al., 2006; Faulk et al., 2007), individual fitness (Bolasina et al., 2006), diet (Papoutsoglou and Lyndon, 2006), and environmental factors. Depending upon fish species and type of digestive enzyme, particularly season and temperature may lead to gradually declining or even to full suppression of digestive enzymes' secretion and activity (Hidalgo et al., 1999; Kolkovski, 2001; Logothetis et al., 2001; Kofuji et al., 2005), including to decrease the digestive contribution of live food organisms themselves (Dabrowski and Glogowski, 1977a, b). In the respect to preceding, Drozd et al. (2010a) observed in *M. fossilis* larvae incubated at lower temperatures (9 and 12 °C), that ingested food particles often leave the digestive tract either only partially predigested (12 °C), or wholly undigested (9 °C). Moreover, ingestion of the first exogenous food even precedes the digestive system's activation (at 9 °C), which occurs simultaneously with the absolute yolk sac depletion (if it ever occurs) (Drozd et al., 2010a).

The optimal temperatures for *M. fossilis* larvae survival lie according to results shown by Drozd et al. (2010a) in the range of 15–21 °C (24 °C is considered by them a suboptimal temperature, as survival rate a couple of days post-onset of exogenous feeding declined appreciably), i.e. within a zone of

lower temperatures in comparison to maximal growth (which spans from 21 to 24 °C, as determined by the fastest achievement of maximum size), as in striped bass *Morone saxatilis* (Walbaum) (Morgan et al., 1981), white perch *Morone americana* (Gmelin) (Morgan and Rasin, 1982) and rainbow trout *Oncorhynchus mykiss* (Walbaum) (Kato and Kamler, 1983).

## Conclusion

In summary, Drozd et al. (2010a) revealed, that in *M. fossilis* the early life history (from egg activation to onset of exogenous feeding) is overall within a viable temperature range (9–24 °C and 15–24 °C for the embryonic and larval periods, respectively) a truly thermal-independent process in terms of yolk sac utilization efficiency [as in *C. nasus*; Kamler (1998)] and specimen outward appearances, as individuals achieve similar size and developmental stage at all key events regardless of temperature. By contrast, however, there is a time aspect in evaluating the onset of any crucial event (temporal prolongation at lower temperatures and vice versa), as in *C. nasus* (Kamler, 1998), and assessing any rate (survival, developmental and growth rate including temperature $Q_{10}$ coefficients).

In conclusion, *M. fossilis* (Drozd et al., 2009, 2010a), like the related species *C. taenia* (Bohlen, 2003), is a typical warm-mesothermic species (classification according to Wieser, 1991). Based on a combination of survival and energetic performance, 15–24 °C and 15–21 °C seem to be optimal temperatures for *M. fossilis* egg incubation and rearing of yolk-feeding larvae, respectively (Drozd et al., 2010a). Nevertheless, as shown by Drozd et al. (2009, 2010a), as well as by Kotlyarevskaja's (1967) observation of normally developing *M. fossilis* eggs at 7–7.5 °C in the wild (with oxygen concentration 3.2–9.2 ml.l⁻¹), *M. fossilis* is commonly able to tolerate such low temperatures as do coldwater fishes [e.g. most salmonids, esocids and percids, as summarized by Kamler (2002)] for relatively long periods as a response to periodic water temperature disturbances at the spawning localities during the spring mating season.

Further studies, which should be undertaken not only under laboratory conditions, but in the nature too, should be directed to understanding of interactions among development, behaviour and exogenous factors influencing early-life history of *M. fossilis*, following  purposes of effective conservation or fisheries management of the natural population of this hidden living fish species.

## PLOIDY LEVELS IN WEATHERFISH *M. fossilis* (L.)

### Sympatric occurrence of polyploid *M. fossilis*

A bisexually reproducing fish population with regular gametogenesis cycles behaves as a biological diploid regardless of its chromosome number (Ráb and Collares-Pereira, 2004). Nevertheless, sporadic literature data presented so far consider *M. fossilis* with 100 chromosomes (Raicu and Taisescu, 1972; Boroń, 2000; Ene and Suciu, 2000), but two studies (Vujošević et al., 1983; Palíková et al., 2007), as well as data presented by Ráb et al. (unpublished data, pers. comm.) from the Elbe (Czech Republic) and Moscow (Russia) River, reported on specimens with 2n = 50 chromosomes. To distinguish such situations, specimens with 50 chromosomes are named as diploids and specimens with 100 chromosomes as tetraploids. No report on *M. fossilis* ever mentioned sympatry of such tetraploid and diploid individuals within one natural locality.

However, Drozd et al. (2010b) found in floodplain area pools on upper reaches of the Lužnice R. [Vltava (Elbe) R. basin, the North Sea drainage, Czech Republic] not only a sympatric occurrence of tetraploid

and triploid individuals but also an existence of triploid and intermediate aneuploid individuals, i.e. ploidy levels recognized by Arai et al. (1991) and Zhang and Arai (1999a, 2003) in another species, *M. anguillicaudatus*.

Frequency of occurrence of particular ploidy levels in combination with their sex ratios suggested a prevailing majority of tetraploids (66% of specimens studied) in equal proportion for both sexes. Nevertheless, triploids and intermediate aneuploids were also notably represented within the wild *M. fossilis* population studied (c. 17% of specimens for each), and the two groups also differed in their sex ratios (Drozd et al., 2010b).

The specimens of *M. fossilis* thus could be safely distinguished by their different ploidy levels as revealed by karyotyping, relative DNA content by flow cytometry, haploid nuclear DNA content (the haploid C-value) by Feulgen image densitometry, as well as by dimensional characteristics of their erythrocyte nuclei. Ploidy ratio of tetraploid/triploid individuals for all variables studied in *M. fossilis* by Drozd et al. (2010b) oscillated round about value 1.20 on average (based on data in Table 1), similarly to findings of Arai et al. (1991), Gao et al. (2007) or to results presented by Drozd et al. (2010b) in *M. anguillicaudatus* standards. Only nuclear volume represented an exception from this rule, it reached almost 1.50 fold value in tetraploid individuals if compared to triploid ones, because of the mathematical formula used, which was originally defined for ideal oblate spheroids or ellipsoids.

The haploid C-value of erythrocytes from tetraploid individuals (2.02 ± 0.09 pg.nucleus-1) evidenced by Drozd et al. (2010b) is slightly lower value compared to the database record for *M. fossilis* specimens with 100 chromosomes [2.60 pg.nucleus$^{-1}$; Timofeeva and Kaviani (1964) quoted in www.genomesize. com by Gregory, 2009] which is based upon a different analytical method (biochemical analysis of sperm cells).

A possible origin of triploids could be an addition of a sperm genome to an unreduced diploid egg, as in the case of *M. anguillicaudatus* (Zhang et al., 1998; Itono et al., 2007; Morishima et al., 2008a), or in the Cobitis species complex (Vasiľev et al., 1989; Janko et al., 2007). Nor can hypothetical crossing of *M. fossilis* forms with 2n = 50 and 2n = 100 chromosomes be excluded. Survival, spreading especially reproduction of triploids in a natural population of *M. fossilis* remains unknown, because fertility of triploid *M. fossilis* has not yet been investigated (Drozd et al., 2010b). However, the balanced sex ratio 1:1 in triploids might suggest their fertility and usual bisexual reproduction as described e.g. for batura toad *Bufo baturae* Stoeck, Schmidt, Steinlein and Grosse (Stöck et al., 2002).

In *M. anguillicaudatus*, however, Zhang et al. (1998) found only sterile triploid males and Oshima et al. (2005) found triploid males mostly producing unreduced sperm incapable of fertilization and only negligible concentration of haploid sperm. On the other hand, natural clone-derived *M. anguillicaudatus* triploids form mainly 1n eggs, sometimes 2n, 3n and aneuploid eggs (Oshima et al., 2005; Morishima et al., 2008b). Another type of wild *M. anguillicaudatus* triploid (originating from tetraploid x diploid mating) produced 1n and unreduced 3n eggs simultaneously (Zhang et al., 1998).

Alternatively, asexual gynogenetic reproduction of triploids allowing survival and spreading independently on a bisexually reproducing lineage could explain this phenomenon, as in the case of spined loaches Cobitis sp. (Janko et al., 2007).

Intermediate aneuploids found by Drozd et al. (2010b) showed true intermediate character between triploid and tetraploid, demonstrated by chromosome number and relative DNA content (Figure 1), and were thus probably unaffected by chromosome losses during preparation. However, with Feulgen image analysis densitometry, intermediate aneuploids were often hidden within tetraploids. With erythrocyte dimension analysis, they were often hidden within triploids. According to Zhang and Arai (1999b), such individuals described as hyper-triploids probably arose from fertilization of a diploid egg of a tetraploid female by an aneuploid sperm of an artificially induced triploid male. Data shown by Drozd et al. (2010b), according with conditions discovered by Arai and Inamori (1999) and Zhang and

Arai (1999a, b) in *M. anguillicaudatus*, thus suggested a certain tolerance of *M. fossilis* to polyploidy as well as to aneuploidy. Consequently, further karyotype studies are therefore highly desirable. Findings of intermediate aneuploids by Drozd et al. (2010b) might indicate hypothetical fertilization capacity of natural triploid individuals in *M. fossilis*, as well as possible hybridization of sympatrically living triploid and tetraploid individuals.

## Conclusion

In summary, results shown by Drozd et al. (2010b) supported by the previously published data on karyotype of *M. fossilis*, hypothesize an existence of a diploid-aneuploid-polyploid complex in *M. fossilis* throughout the Europe. By analogy with data summarized by Arai (2003) for *M. anguillicaudatus*, sympatric occurrence of spontaneous triploid, tetraploid and intermediate aneuploid individuals shows an obvious evidence of possible contact zones between genetically different lineages, and thus also migth support a hypothesis about the existence of at least two distinct genetical lineages (2n = 50 or 100) or even different species within European weatherfish *M. fossilis* and their reproductive contacts. This hypothesis is also highlighted by a relative high frequency of occurrence of natural triploids and intermediate aneuploids within floodplain area of the Lužnice R. (Drozd et al., 2010b). In addition, data presented by Drozd et al. (2010b), also support a hypothesis proposed by Bohlen et al. (2007) on existence of at least an additional refuge (smaller in size, more Western and Northern situated) to the Danubian one, for *M. fossilis* survival over glacial ages, proposed on the basis of haplotype richness of *M. fossilis* within this area (Bohlen et al., 2007; Mendel et al., 2008). *M. fossilis* originating from the Lužnice R. and its tributaries, thus could prosper to colonization of some parts of present distribution range (especially the western and northern parts) at least after termination of the last ice age.

Nevertheless, all theories about origin of the triploid form are based on at least putative existence of diploid individuals. Therefore not finding any diploid individual by Drozd et al. (2010b) is considered surprising but not strange. An explanation for this phenomenon may be found either in capture unability (i.e. in sampling deficiency) due to very low frequence of occurrence of diploids, or probably in their real absence at the examined localities in floodplain area of the Lužnice River. Typical *M. fossilis* localities (Pekárik et al., 2008) are dynamic ecosystems opened for fish migration (Meyer and Hinrichs, 2000) with fluctuating and often unfavourable conditions, both abiotic (oscillating temperature and oxygen concentration; presence of ammonium, methane, sulphide) and biotic (presence of predator), representing periodic strong bottleneck effect and genetic drift. Just sympatrical occurrence of *M. fossilis* and Northern pike *Esox lucius* L., i.e. a typical fish predator feeding also on *M. fossilis*, in combination with fluctuating water level (predator´s ability/inability to find a prey) may be considered one of the most important factors significantly affecting abundance of natural *M. fossilis* populations (Musil and Drozd – unpublished telemetric data) and possibly also the composition of population ploidy levels within the process of *M. fossilis* population recruitment.

However, we have not yet sufficient data set to draw complete conclusions about ploidy distribution, increase and elevation of particular ploidy levels in *M. fossilis*. Therefore, experimental crossing of individuals with different ploidy levels and subsequent analyses of progeny combined with molecular cytogenetic approach is to be used for specimens from larger area in order to get more resolution in terms of ploidy level and species status. In context of this suggestion and according to forming of putative mosaic individuals (observed in *M. anguillicaudatus* by Zhang and Arai, 2003 and Itono et al., 2007), it is recommended to use for such cytogenetic studies samples of as many tissues as possible (fin clip from different fins, blood, eggs, sperm) using non-invasive tissue sampling because of EN – VU conservation status throughout the Europe (Lelek, 1987).

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## ENGLISH SUMMARY

### Study of selected population parameters of weatherfish *Misgurnus fossilis* (Cypriniformes, Cobitidae): early life history and status of ploidy in fish from Lužnice River floodplain area

*Bořek Drozd*

Weatherfish *Misgurnus fossilis* (L.) belongs to fishes crucial for explaining river inundation areas' importance in respect to successful fish reproduction, as well as to distribution and dynamics of early developmental stages. In order to predict wild population dynamics and life demands of weatherfish for purposes of effective conservation management, the principal goals of the present Ph.D. thesis were an experimental evaluation of thermal sensitivy of early life history of *M. fossilis* (chapter 2), as well as elucidation the ploidy levels question (chapter 3) in natural *M. fossilis* population inhabiting the floodplain area of the Lužnice River.

Early life history of *M. fossilis* (from egg activation to absolute yolk sac resorption) as evaluated at wide temperature range (9–36 °C, interval 3 °C) is overall within a viable temperature range for embryonic (9–24 °C) and larval (15–24 °C) periods. This is a truly thermal-independent process in terms of specimen outward appearances, as similar size and developmental stage were achieved at each of the key events regardless of temperature, and of yolk sac utilization efficiency [within circum-(sub) optimal temperature range]. There is by contrast, however, a time aspect in evaluating the onset of any crucial event (temporal prolongation was observed at lower temperatures) and in assessing any rate (survival, developmental and growth rate including also temperature $Q_{10}$ coefficients).

*M. fossilis* embryonic development is theoretically arrested at ca 8.5 °C; hatching ($H_{50}$) occurs at $D°_{eff} = 29$; larval development is theoretically arrested at ca 7 °C; and the yolk sac is full resorbed after the next 91 effective day-degrees. The lowest viable temperatures were computed at 9.11 and 7.77 °C in terms of embryonic and larval survival, respectively. In addition, it may be supposed that: (a) length might determine the age at hatching, rather than the age at hatching is determining the hatching length; and (b) growth within eggs is most likely unequal, and thus a synergistic effect of both embryonic growth and temperature probably determines the hatching time in *M. fossilis*.

Temperatures within the survival and developmental optima (15–24 °C and 15–21 °C for embryonic and larval period, respectively) accelerated development in terms of incubation, as well as hatching period duration and observation of the digestive system activation (Ac), onset of mixed (S) and exogenous (Re) feeding during different intervals of the embryonic and larval periods in a uniform (inversely proportional) way.

An absolute lethal effect of higher temperatures (27–36 °C) manifested itself already during early cleavage [all eggs died in the stages of 4 blastomers (30–36 °C) or 8 blastomers (27 °C)], but in the case of lower temperatures (9 and 12 °C) this was not expressed until Re.

The two-stage curve (with an inflection region shortly after S) provided a useful model for evaluating growth (assessed in terms of total length increase) and other morphometric characteristics. However, accelerated development of eyes as one of the sensory organs (evaluated in terms of orbital length increase) was observed as a response to environmental needs (timely food detection, predator avoidance).

Regardless of temperature, larvae did not fully deplete the yolk sac, i.e. they do not start to utilize the exogenous nutrition only, until the gut had become a functional additional respiratory organ.

Based on a combination of survival and energetic performance, 15–24 °C and 15–21 °C seem to be optimal temperatures for *M. fossilis* egg incubation and rearing of yolk-feeding larvae, respectively.

*M. fossilis* is thus considered a typical warm-mesothermic species. Nevertheless, *M. fossilis* is commonly able to tolerate such low temperatures as do coldwater fishes (e.g. most salmonids, esocids and percids) for relatively long periods as a response to periodic water temperature disturbances at the spawning localities during the spring mating season.

A sympatric occurrence of tetraploid and triploid *M. fossilis* individuals, as well as an existence of triploid and intermediate aneuploid weatherfish individuals [i.e. ploidy levels up to now recognized in related species oriental weatherfish *Misgurnus anguillicaudatus* (Cantor) only], were found. Frequency of occurrence of particular ploidy levels in combination with their sex ratios suggested a prevailing majority of tetraploids (66%) in equal proportion for both sexes. Nevertheless, triploids (17%) and intermediate aneuploids (17%) were also notably represented. Sex ratio of triploids was 1:1 while that of aneuploids was skewed at 3:1 for males. Ploidy ratio of tetraploid/triploid individuals oscillated commonly round about value 1.20 on average (but excluding nuclear volume). The haploid C-value of erythrocytes from tetraploid individuals (specimens with 100 chromosomes) was $2.02 \pm 0.09$ pg.nucleus$^{-1}$.

The equal proportion of sex ratio in triploids might suggest their fertility and usual bisexual reproduction. Alternatively, asexual gynogenetic reproduction of triploids allowing survival and spreading independently on a bisexually reproducing lineage could explain this phenomenon too. Intermediate aneuploids showed true intermediate character between triploid and tetraploid, demonstrated by chromosome number and relative DNA content. Their findings might indicate hypothetical fertilization capacity of natural triploid individuals in *M. fossilis*, as well as possible hybridization of sympatrically living triploid and tetraploid individuals.

The present data (boosted by the previously published data on karyotype): (a) suggest a certain tolerance of *M. fossilis* to polyploidy as well as to aneuploidy; (b) hypothesize and existence of a diploid-aneuploid-polyploid complex in *M. fossilis* throughout the Europe; (c) support a proposed hypothesis on existence of at least an additional refuge to the Danubian one for *M. fossilis* survival over glacial ages (proposed on the basis of haplotype richness in available evidence). *M. fossilis* originating from the Lužnice R. and its tributaries, thus could prosper to colonization of some parts of present distribution range at least after termination of the last ice age.

Sympatric occurrence of spontaneous triploid, tetraploid and intermediate aneuploid individuals commonly shows an obvious evidence of possible contact zones between genetically different lineages, and thus present data also migth support a hypothesis about the existence of at least two distinct genetical lineages (2n = 50 or 100 chromosomes) or even different species within *M. fossilis* and their reproductive contacts.

However, experimental crossing of individuals with different ploidy levels and subsequent analyses of progeny combined with molecular cytogenetic approach is to be used for specimens from larger area in order to get more resolution in terms of ploidy level and species status.

## CZECH SUMMARY

### Studie vybraných populačních parametrů piskoře pruhovaného, *Misgurnus fossilis* (Cypriniformes, Cobitidae): raná ontogeneze a úroveň ploidie u ryb ze záplavového území Lužnice

*Bořek Drozd*

Piskoř pruhovaný, *Misgurnus fossilis* (Linné, 1758), představuje jeden z klíčových rybích druhů z hlediska pochopení a vysvětlení významu záplavových území řek z hlediska úspěšné reprodukce i rozšíření a dynamiky raných ontogenetických stádií říčních druhů ryb. Hlavními cíli předložené doktorské disertační práce vedoucími k vytvoření účelného managementu druhové ochrany piskoře pruhovaného, díky předpovězení nároků tohoto druhu na podmínky životního prostředí i jeho populační dynamiky, se tak staly:

1) Experimentální zhodnocení závislosti raného ontogenetického vývoje piskoře pruhovaného na teplotě (kapitola 2).

2) Objasnění problematiky ploidní úrovně divokých populací piskoře pruhovaného obývajících inundační území řeky Lužnice (kapitola 3).

Raný ontogenetický vývoj (od oplození po přechod na exogenní potravu) piskoře pruhovaného, který byl hodnocen v teplotním rozmezí 9–36 °C (s intervalem 3 °C), představuje v intervalu tolerovaných teplot pro embryonální (9–24 °C) a larvální (15–24 °C) periodu teplotně nezávislý proces z hlediska: a) vnějšího vzhledu jedince (stejné dosažené vývojové stádium a celková délka těla v jednotlivých klíčových bodech ontogeneze bez ohledu na teplotu) i b) účinnosti konverze masy žloutkového váčku do tělních tkání [v rozmezí (sub)optimálních teplot]. Tato skutečnost však neplatí z hlediska časového určení nástupu jednotlivých klíčových ontogenetických momentů (pozorována prolongace při nízkých teplotách) a stanovení hodnot přežívání, vývojové a růstové rychlosti (včetně hodnot teplotních koeficientů $Q_{10}$).

Embryonální vývoj je teoreticky zastaven při teplotě cca 8,5 °C a líhnutí nastává po uplynutí 29 efektivních denních stupňů. Naproti tomu larvální vývoj je teoreticky zastaven při teplotě cca 7 °C a žloutkový váček je plně resorbován po uplynutí dalších 91 efektivních denních stupňů. Nejnižší teplota pro přežití byla matematicky stanovena na 9,11 °C pro embryonální a 7,77 °C pro larvální periodu. Pro embryonální vývoj může být dále předpokládáno: a) velikost jedince determinuje věk při líhnutí z jikry (spíše než že by věk předurčoval délku při líhnutí), b) růst uvnitř jikry se jeví jako nestejnoměrný, souhlasně působící vliv embryonálního růstu a teploty tedy pravděpodobně určuje čas líhnutí.

Při optimálních teplotních podmínkách pro přežívání i vývoj (tj. 15–24 °C pro embryonální a 15–21 °C pro larvální periodu) je vývoj akcelerován během různých etap embryonální a larvální periody shodným způsobem, tj. délka inkubační doby i doby líhnutí, stejně tak jako aktivace trávicí soustavy, nástupu mixogenní a exogenní výživy klesají s rostoucí teplotou.

Letální efekt vysokých teplot (27–36 °C) se projevuje již během rýhování oplozeného vajíčka (všechna vajíčka inkubovaná při 27 °C či 30–36 °C umírají ve stádiu 8 popř. 4 blastomér). Letalita nízkých teplot (9 a 12 °C) se však projevuje až po definitivním přechodu na exogenní výživu, tj. po úplném vyčerpání energetických zásob žloutkového váčku.

Z hlediska hodnocení průběhu růstu (nárůst celkové délky těla v čase) i ostatních morfometrických znaků se jeví jako nejvýhodnější použití dvojstupňového modelu (s výrazným inflexním bodem objevujícím se po přechodu na mixogenní výživu). Oko jako jeden ze smyslových orgánů jeví silně akcelerovaný vývoj v důsledku zvýšených nároků na jedince (včasná detekce potravy a predátora).

Bez ohledu na teplotu, k přechodu na exogenní výživu dochází teprve až v momentu, kdy střevo

začíná plnit svou funkci přídavného dýchacího orgánu.

Na základě kombinace výsledků týkajících se míry přežívání a teplotně podmíněné energetické výkonnosti, rozmezí teplot 15–24 °C a 15–21 °C se jeví jako optimální teplotní podmínky pro inkubaci jiker a kultivaci larev piskoře pruhovaného. Piskoř pruhovaný je tak z hlediska teplotní preference považován za typický mezotermní rybí druh, který je však schopen tolerovat po relativně dlouhou dobu i velice nízké teploty jako studenomilné druhy ryb (většina lososovitých, dále pak štikovité a okounovité ryby). Tuto skutečnost lze považovat za vývojovou adaptaci piskoře pruhovaného na nestabilní teplotní podmínky (včetně nízkých teplot) na trdlištích během jarního výtěrového období.

V rámci přirozených populací obývajících inundační území řeky Lužnice byl potvrzen sympatrický výskyt tetraploidních, triploidních a aneuploidních jedinců piskoře pruhovaného. Triploidie a aneuploidie byla u tohoto druhu nalezena zcela poprvé. Frekvence výskytu jednotlivých ploidních úrovní v kombinaci s pohlavím studovaných jedinců prokázala převahu tetraploidních jedinců (66 %) s poměrem pohlaví 1 : 1. Avšak i triploidní (17 %) a aneuploidní (17 %) jedinci byli významně zastoupeni ve studované populaci. Poměr pohlaví byl u triploidů 1 : 1, u aneuploidů pak 3 : 1 ve prospěch samců. Ploidní koeficient tetraploidních/triploidních jedinců kolísal v průměru kolem hodnoty 1,20 (toto však neplatilo pro objem jádra). Haploidní objem jaderné DNA erytrocytů dosahoval u tetraploidních jedinců (jedinci se 100 chromosomy) průměrné hodnoty 2,02 ± 0,09 pg/jádro.

Vyrovnaný poměr pohlaví u triploidů by mohl být vysvětlen dvěma způsoby: a) plodností těchto jedinců a obvyklou bisexuální reprodukcí, nebo b) asexuální gynogenetickou reprodukcí triploidů, jež by umožnila zachování a šíření této ploidní úrovně nezávisle na bisexuálním způsobu reprodukce.

Aneuploidní jedinci představují skutečný přechodný stupeň mezi triploidií a tetraploidií z hlediska počtu chromosomů a relativního obsahu DNA. Jejich nález tak podporuje myšlenku: a) alespoň hypotetické možnosti pohlavního množení u triploidních jedinců, b) možné hybridizace sympatricky žijících triploidních a tetraploidních jedinců.

Tato část výsledků doktorské disertační práce (podpořená údaji z literatury): a) naznačuje značnou toleranci piskoře pruhovaného jako druhu k polyploidii i aneuploidii, b) přináší důkaz o existenci diploidního-aneuploidního-polyploidního komplexu uvnitř druhu *M. fossilis* v rámci Evropy, c) podporuje jinými autory navrhovanou hypotézu (založenou na základě množství nalezených různých haplotypů) o existenci přinejmenším jednoho dalšího refugia (vedle hlavního refugia v povodí Dunaje) pro přežití druhu během dob ledových. Na základě toho lze tedy usuzovat, že piskoři pruhovaní pocházející z povodí řeky Lužnice tak mohli kolonizovat některé z dalších částí současného areálu rozšíření tohoto druhu. K tomuto jevu pak mohlo dojít přinejmenším jednou, a to po skončení poslední doby ledové.

Sympatrický výskyt spontánních triploidních, tetraploidních a aneuploidních jedinců obvykle svědčí o přítomnosti možných kontaktních zón mezi geneticky odlišnými liniemi. Proto výsledky prezentované v doktorské disertační práci mohou také podporovat hypotézu existence přinejmenším dvou odlišných genetických linií (2n = 50 nebo 100 chromosomů), nebo dokonce odlišných druhů, v rámci druhu *M. fossilis* a jejich reprodukčního kontaktu.

Aby však bylo skutečně možné objasnit či vyřešit problematiku ploidní úrovně a druhového statusu piskoře pruhovaného, je bezpodmínečně nutné využít cytogenetických technik v rozsáhlých studiích jedinců pocházejících z širší oblasti výskytu druhu, v kombinaci s experimentálním křížením jedinců různých ploidních úrovní a následnou analýzou takto získaného potomstva.

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5) 86/2006/P-VÚRH Effect of water temperature on early ontogeny in weatherfish (*Misgurnus fossilis*), funded by the Grant Agency of the USB in České Budějovice (2007, coordinator: M.Sc. Bořek Drozd)

6) GAJU 047/2010/Z – Breeding and environmental aspects of aquaculture and hydrocoenoses (2010–2012, leader: Assoc. Prof. Dipl.-Ing. Martin Flajšhans, Dr.rer.agr.)

7) Czech-Russian bilateral project (KONTAKT programme) No. ME853 New fish species and breeding methods in aquaculture, funded by the Ministry of Education of the Czech Republic (2006–2008, coordinator: Assoc. Prof. Dipl.-Ing. Jan Kouřil, Ph.D.)

## LIST OF PUBLICATIONS

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### ABSTRACTS AND CONFERENCE PROCEEDINGS

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**Drozd, B.**, 2006. Pharyngeal dentition in asp: a new model for early stages of pharyngeal teeth development? In: "Growth factors in craniofacial development and repair", International Meeting of COST Action B23, 4th–7th May 2006, Helsinki, Finland, p. 41.

# TRAINING AND SUPERVISION PLAN DURING STUDY

| Name | Bořek Drozd |
|---|---|
| Laboratory | 2006–2009: Department of Hydrobiology and Aquaculture (RIFCH USB) |
| | 2009–2010: Laboratory of Fish Reproduction and Intensive Culture (FFPW USB) |
| | Since 2010: Laboratory of Artificial Propagation of Fish (FFPW USB) |
| Supervisor | Assoc. Prof. Dipl.-Ing. Jan Kouřil, Ph.D. |
| Period | 2nd October 2006 until 14th September 2011 |

| Ph.D. courses | Year |
|---|---|
| Pond culture | 2007 |
| Biostatistics | 2007 |
| Fish genetics | 2008 |
| Applied hydrobiology | 2008 |
| English | 2009 |

| Scientific seminars | Year |
|---|---|
| Scientific seminar at USB RIFCH 1/2007 | 2007 |
| Scientific seminar at USB RIFCH 11/2007 | 2007 |
| Scientific seminar at USB RIFCH 2/2009 | 2009 |
| Scientific seminar at USB FFPW 11/2009 | 2009 |
| Scientific seminar at USB FFPW 12/2010 | 2010 |

| International conferences | Year |
|---|---|
| Bodrogköz Conference, 23rd January, Budapest, Hungary (poster) | 2007 |
| Revitalisation of Small watercourses, 5th–6th March, Budapest, Hungary (poster) | 2007 |
| Environmental Accounting and Sustainable Development Indicators 2007 – Environmental Accounting and Reporting at Micro Level, 23rd–25th May, Prague, Czech Republic (poster) | 2007 |
| 8th International Congress of Vertebrate Morphology, 16th–21th July, Paris, France (oral presentation) | 2007 |
| XII. European Congress of Ichthyology, 9th–13rd September, Dubrovnik – Cavtat, Croatia (oral presentation) | 2009 |
| XIII. European Congress of Ichthyology, 6th–12th September, Klaipeda, Lithuania (poster) | 2007 |
| Diversification in Inland Finfish Aquaculture (DIFA), 16th–18th May, Pisek, Czech Republic (poster) | 2011 |

| | |
|---|---|
| Aquaculture of Europe and Asia: Realities and prospects for development and cooperation, 1th–7th August 2011, Ulan-Ude, Baikal, Russia (oral presentation) | 2011 |

| **National conferences** | **Year** |
|---|---|
| X. Česká ichtyologická konference s mezinárodní účastí, 26th–27th June, Prague, Czech Republic (oral presentation) | 2007 |
| XII. Česká ichtyologická konference s mezinárodní účastí, 19th–20th May, Vodňany, Czech Republic (oral presentation) | 2010 |

| **Foreign stays during Ph.D. study at FFPW (RIFCH)** | **Term** | **Year** |
|---|---|---|
| Prof. Hannu Lehtonen (University of Helsinki, Faculty of Biosciences, Dpt. of Biological and Environmental Sciences) & Dr. Jukka Ruuhijärvi (Finnish Game and Fisheries Research Institute of Helsinki – Evo field station), Finland. Research topic: Fish biomanipulation in the small forest lakes. | 3 months | 2009 |

# CURRICULUM VITAE

## PERSONAL INFORMATION

| | |
|---|---|
| Name: | **Bořek Drozd** |
| Degree: | **MSc. (RNDr.)** |
| Born: | 19th June 1983, Kutná Hora |
| Nationality: | Czech, Czech Republic |
| Marital Status: | Married |
| Contacts: | University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters (USB FFPW), Institute of Aquaculture, Laboratory of Artificial Propagation of Fish, Husova třída 458/102, 370 05 České Budějovice, Czech Republic, e-mail: drozd@frov.jcu.cz, phone: +420/387 774 607 |

## EDUCATION

| | |
|---|---|
| 2007 | Charles University in Prague, Faculty of Science – rigorous exam: RNDr. |
| Since 2006 | USB in České Budějovice, FFPW, Ph.D. study in program Zootechnics (specialization: Fishery) |
| 2001–2006 | Charles University in Prague, Faculty of Science – M.Sc. |

## APPOINTMENTS

| | |
|---|---|
| 2006–2010 | USB in České Budějovice, FFPW, researcher, technician |
| Since 2010 | USB in České Budějovice, FFPW, academic worker |

## RESEARCH INTERESTS

Early fish life history, morphology, ploidy level, biology of protected species

## INTERNATIONAL PROFESSIONAL EXPERIENCE

| | |
|---|---|
| 2008 | Study training stay at the University of Helsinki & Finnish Game and Fisheries Research Institute of Helsinki, Finland |
| 2008–2006 | Regular short-term research missions to Hungary (cooperation with Corvinus University of Budapest & Szent Istvan University, Gödöllö) |
| 2005 | Study stay at the Corvinus University of Budapest & Balaton Limnological Research Institute of the HAS, Hungary |

## LANGUAGE SKILLS

Czech (native), English (intermediate), German (intermediate)

## PROJECT PARTICIPATION

Effect of water temperature on early ontogeny in weatherfish (*Misgurnus fossilis*) (funded by the Grant Agency of the USB in České Budějovice, 2007) (coordinator)

Environmentally and hormonally induced reproduction, anaesthesia, early life history and breeding of selected endangered and commercially imported fishes (Czech-Russian bilateral project, KONTAKT programme, funded by the Ministry of Education of the Czech Republic, 2010–2012, coordinator: Assoc. Prof. Dipl.-Ing. J. Kouřil, Ph.D.) (co-investigator)

Stream revitalization according to the European water framework directive (Czech-Hungarian bilateral project, KONTAKT programme, supported by the Ministry of Education of the Czech Republic, 2006–2007, coordinator: MSc. M. Švátora, CSc.) (co-investigator)

## AWARDS, SCHOLARSHIPS

| | |
|---|---|
| 2008 | ERASMUS scholarship: University of Helsinki, Finland |
| 2005 | CEEPUS scholarship: Corvinus University of Budapest, Hungary |
| 2001 | Winner of the national round of the High school Student Scientific Activity |