RESEARCH PAPER

Early Development of the Asp, *Leuciscus aspius* Pseudobranch - the Histological Study

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Abstract

The postembryonic development of the asp, *Leuciscusaspius* (Linnaeus, 1758) pseudobranch using the technique of histological section and microscopy was done. The morphometricand histological analyses revealed that the morphology of asp pseudobranch changes during early ontogeny of larvae. Histological observations indicated that the pseudobranch was the lamellae-free type just after hatch, then lamellae are fused but the filaments stay free. Generally, the basic structure of the pseudobranch of asp larvae since 14 dph consisted of several rows of parallel lamellae. From 14 dphpseudobranch cells, pillar cells, epithelial cells and lacunar tissue were noted in the asp larvae pseudobranch. Neither chloride cells nor mucus cells were present at this structure, similarly to other fish species possessing covered type of pseudobranch.

Keywords:Cyprinid fish larvae, gill-like structure morphology, ontogeny.

Introduction

The pseudobranch is found in almost all fishes among the known teleostean families, with the exception of a few species belonging to order Anguilliformes, suborder Siluroidei, and all species of genera *Gymnarchus* and *Cobitis* (Wittenberg and Haedrich, 1974). The pseudobranch arises embryologically from the caudal hemibranch of the mandibular gill arch. It is supplied with oxygenated blood and therefore has no respiratory function in adult fish (Stoskopf, 1993). The pseudobranch is a bilaterally paired organ. It is structurally similar to the gill, with filaments and lamellae. In the teleosts the pseudobranch is located in the cranial part of the opercular cavity to which it is attached by one of its sides (Roy *et al*., 1997). Bertin (1958) described three morphological types of pseudobranch in fishes:

I) these with distinguishable lamellae in contact with the water that the lamellae are completely free.

II) those that were covered with the opercular membrane and the connective tissue. This kind can be subcategorized into three types based on its location and its degree of isolation from water:

A- a thin layer of connective tissue due to which lamellae can not be separated but the filaments are free.

B- connective tissue layer thicker than type A due to which both the filaments and the lamellae cannot be separated. The lamellae, rays and the vessels are well organized.

C- a thick layer of connective tissue in which the structure is so covered that all the elements lose their identity.

III) Those that were completely reduced and embedded in the tissue. In this type pseudobranch is separated from the opercular chamber by the folds of connective tissue. This causes sinking of the pseudobranch in the roof of the buccopharynx or opercular cavity.

Different roles of the pseudobranch have been proposed by several authors, for example: secretory function (Parry and Holliday, 1960; Lange, 1972), regulation of the gas exchange at the eye (Wittenberg and Wittenberg, 1962; Wittenberg and Haedrich, 1974) and the blood pH regulation (Berenbrink, 1994; Müller, 1995).

There are only a few papers devoted to pseudobranch histology (Singh *et al*., 1986; King *et al*., 1993; Waser *et al*., 2005; Hamidian and Alboghobeish, 2007; Yang *et al*., 2014). Unfortunately the early development of the

pseudobranch has seldom been investigated.

The aim of this study was to describe the postembryonic development of the asp, *Leuciscusaspius* (Linnaeus, 1758) pseudobranch using the technique of histological section and microscopy.

Material and Methods

Asp larvae were obtained by artificial spawning of fish originating from the lake Sukiel (north-eastern Poland). Gametes were obtained from spawners stimulated as described by Targońska *et al.* (2008). The eggs were incubated 11 days in Weiss jars, at 13°C (±0.5). After hatching, the larvae were moved to a semi-closed laboratory recirculating system, where they were stocked in two 25 dm³ glass tanks. The initial stocking density of larvae was 40 ind.dm⁻³. The system was equipped with biological filtration, aeration and automatic temperature regulation (±0.1°C). The water temperature in the tanks was 25°C. Specimens were exposed to a 12 L:12 D photoperiod. The ammonia content during the whole rearing period was below 0.01 mg dm⁻³ and dissolved oxygen saturation was always above 80%. The larvae were fed three times a day *ad libitum* with freshly hatched *Artemia*sp. nauplii (San Francisco origin – SFO, Argent USA) from 4 dph, which coincided with the start of the free swimming, until the end of the experiment. The rearing tanks were cleaned every morning. A random sample of 20 larvae from each tank was collected every five day from hatching to 24 days post-hatch (dph). They were submitted to growth analysis and 10 specimens were taken for further histological studies.

The sampled larvae were anesthetized with tricainemethanesulfonate (MS-222, Sigma, dose of 150 mg dm⁻³), while those for histological studies were sacrificed by overdosing that anaesthetic. Digital photographs of each specimen were taken using JenopticProgRes® 3 and processed using the ProgRes®CapturePro 2.5 software (Jenoptic, Germany). In each digital photograph, the total body length (TL) was measured to the nearest 0.01 mm. After taking photographs, the weight of each larva (W) was determined to the nearest 0.1 mg (±0.0001 g) with an analytical microbalance KERN ALJ 220-4M. Afterwards, they were fixed in Bouin solution, dehydrated in graded ethanol, embedded in paraffin and cut into serial cross and longitudinal sections (5-8 µm thick) with a Leica RM2265 microtome. Sections were stained according to the Mayers' Haematoxylin and Eosin (HE) procedure for general histomorphological observations. Histological sections were photographed with a Leica DFC420 digital camera (Leica Microsystems Switzerland Ltd.) coupled to a Leica DM 2500 microscope and processed using software designated by the manufacturer (Leica Application Suite v 3.5.0).

Histological analyses were carried out to evaluate the following morphological features of the pseudobranch: shape (free or compacted lamellae), filament number, size (area of the particular filament including lamellar regions, FA), number of lamellae connected to one side of the filament, pseudobranch (PL) and gill (GL) lamellae length (±0.01µm). The relative filament cross-section area (RFA) and relative lamellae length (RL) were calculated according to respective formulas:

$$RFA = 1000\frac{FA}{HA}$$

where:
FA – filament cross-section area
HA – head cross-section area

$$RL = \frac{PL}{GL}$$

where:
PL – pseudobranch lamellae length
GL – gill lamellae length

Statistical differences among age-groups were analysed after one-way ANOVA with post-hoc Tukey test had been performed (P<0.05).

Results and Discussion

The asp larvae showed exponential growth for wet weight (W) and total length (TL) from the beginning of the experiment at 4 dph (W: 2.00 ±0,31mg; TL: 7.50 (±0.15 mm) until the end of the study (24 dph; W: 132.89 ±22.39 mg; TL: 26.05 ±1.35 mm) (Figure 1a). The similar results of the asp larvae rearing were described by Wolnicki (2005) and Kamler and Wolnicki (2006). The shape of both curves is defined most precisely by a power function. The curve for the body length growth was defined by the equation TL = $3.0849x^{0.64}$ (R^2=0.95), while the individual body gain relies on the equation W = $0.0792x^{2.2812}$ (R^2=0.98).

Cross-sectional area of the head (HA) increased with the size of the fish from 0.82 (±0.09) at 4 dphto 9.29 (±1.26) mm² (24 dph) (Figure 1b). The relationship between fish weight and length and fish head cross-section area (HA) is identified by an equation:

$$y = x_1\ 42530 + x_2\ 149339 - 529922,$$

where:
y – area of the head cross section
x_1 – weight of the fish
x_2 – length of the fish
r – 0.98 – correlation coefficient
R^2 – 0.95 determination coefficient
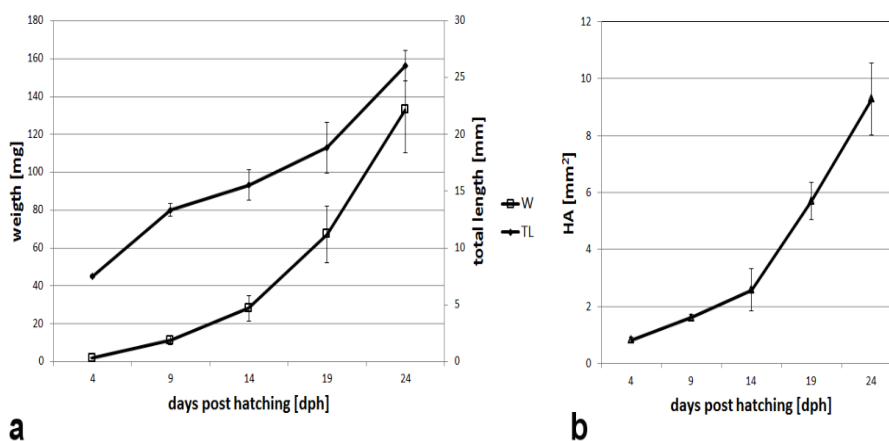P<0.01

The relationship between the pseudobranch

Figure 1. Growth of *Leuciscusaspius* larvae from 4 until 24 days post-hatch (dph); (a) growth in wet weight (left scale) and total length (right scale); (b) growth of head cross-sectional area (HA).

filament cross-section area (FA) and the head cross-section area (HA) was identified by equation:

$$y = x \, 0.005 + 4507.53$$

where
y - pseudobranch filament cross-section area
x - head cross-section area
r – 0.93– correlation coefficient
R^2 – 0.86 determination coefficient
P<0.01

The filament cross-section area (FA) increased from 1.18 x10^{-2} mm^2 to 5,88 x10^{-2} mm^2 during the experiment. However, the relative filament cross-section area (RFA) decreased till 14 dph and next was stable at the same level (Figure 2). Relative lamellae length (RL) slightly decreased during the experiment and extended from 2.51 (±0.45) to 2,02 (±0.26) (Table 1).

The pseudobranch was histologically identifiable at the beginning of the endo-exotrophic stage (4 dph) as a primordial gill-like structure at the anterior part of the opercular cavity very close to the posterior zone of the eyeball (Figure 3). The pseudobranch of the youngest fish (4 dph) contained only one filament on each fish side (TabLE 1). Santamaría *et al.* (2004) observed the pseudobrach anlage in *Dentexdentex* (Linnaeus, 1758) larvae at the beginning of endo-exotrophic period (4 dph). Dunayevskaya *et al.* (2012) described pseudobranch anlage appearance in *Labrusbergylta* (Ascanius, 1769) larvae by 9 dph.*Pagrusauriga* (Valenciennes, 1769) larvae showed the cartilaginous frameworks of pseudobranch at 6 dph (Sánchez-Amaya *et al.* 2007). At 9 dph the asp pseudobranch was composed of free lamellae (Figure 4a). At 4 dph the maximum length of pseudobranch lamellae varied from 69.9 to 81.03 μm and the average number of pseudobranch lamellae per filament was 4.37 ±0.48 (Tab. 1). In older asp larvae (from 14 dph) the pseudobranch was visible as a few bud-like structures (filaments) (Figure 4b, c, d).

Filaments consisted of several rows of parallel lamellae fused to each other throughout their length by a thin connective tissue. The lamellae comprised a central blood capillary that formed a mesh work and surrounded by pseudobranchial cells. The interlamellar space was almost completely reduced and filled by a lacunar tissue as in *Glossogobiusgiuris* (Hamilton, 1822) (Singh *et al.*, 1986), Atlantic cod, *Gadusmorhua* (Linnaeus, 1758) (Morrison, 1993), rainbow trout, *Oncorhynchusmykiss* (Walbaum, 1792) (Mattey *et al.*, 1978; Waser *et al.*, 2005), *Ctenopharyngodonidella* (Valenciennes, 1844) (Hamidian and Alboghobeish, 2007), *Leuciscusidus*(Linnaeus, 1758) (Fisher-Scherl and Hoffmann, 1986). The number of filaments increased during the fish development and varied up from 3 to 5 at the end of the experiment (Tab. 1). In *D. dentex*the second pair of filaments was visible by 6 dph and lamellar primordia could be seen at the end of the endo-exotrophic stage (Santamaría *et al.*, 2004). The third pair of filaments in *D. dentex*larvae was observed between 11 and 22 dph as well as lamellar structure development (Santamaría *et al.*, 2004). The mean number of the asp pseudobranch lamellae per filament increased to 14.85 ± 2.40 at the end of the experiment (24 dph) (Tab. 1). The similar tendency was noted at the larvae of some other studied species (Santamaría *et al.*, 2004; Sánchez-Amaya *et al.*, 2007; Dunayevskaya *et al.*, 2012).

Different types of pseudobranchs have been observed according to the seawater or freshwater habitat of fish. In many freshwater teleosts, the pseudobranch is small and is covered by the opercular epithelium (Roy *et al.*, 1997). This type is referred to as a "glandular pseudobranch" (i.e. belongs to type III in the Bertin's classification) because its lamellae exhibit no contact with the external medium. On the other hand, the pseudobranch is a much more impressive organ (i.e. belongs to type I in the Bertin's classification) in the great majority of marine fishes (Wittenberg and Haedrich, 1974; Laurent and Dunel-
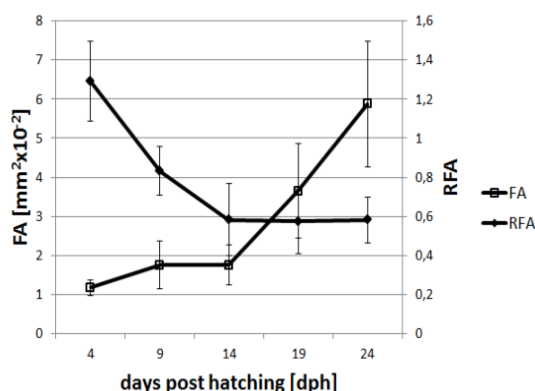
Figure 2. The filament cross-sectional area (FA, left scale) and the relative filament cross-sectional area (RFA, right scale) of the *Leuciscusaspius* larvae pseudobranch from 4 until 24 days post-hatch (dph).

Table 1. Development of asp (*Leuciscusaspius*) larvae pseudobranch from 4 until 24 days post-hatch (dph). Relative lamellae length (RL) calculated according the formula in Material and Methods; data with the same letter index do not differ statistically, ($P < 0.05$)

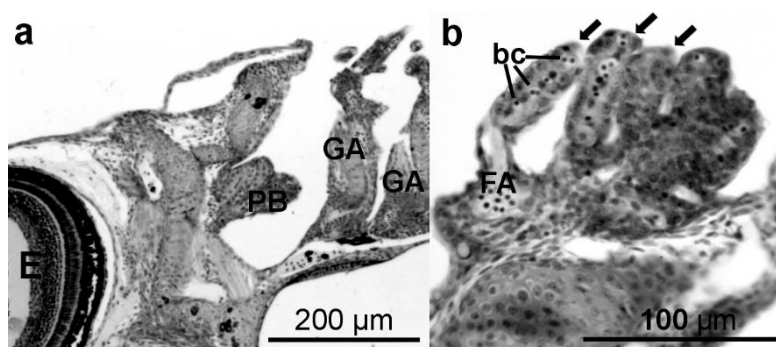| Age of fish (dph)	Number of filaments per one fish side	Number of lamellae per filament	Lamellae length (μm)		Relative lamellae length (RL)
	scope	scope	mean (±SD)	mean (±SD)	mean (±SD)
4	1	4-5	4.37 (±0.48)	70.24 (±9.72)	2.64 (±0.62)[a]
9	2-3	4-9	5.35 (±1.52)	155.92 (±31.00)	2.68 (±0.47)[a]
14	3-4	4-13	6.60 (±1.97)	133.42 (±21.09)	2.51 (±0.45)[a]
19	3-4	7-13	9.95 (±1.74)	167.62 (±21.88)	2.02 (±0.26)[b]
24	3-5	10-19	14.85 (±2.40)	186.14 (±24.95)	2.20 (±0.41)[b]

Figure 3. Longitudinal section by *Leuciscusaspius* opercular cavity at 4 dph; (a) pseudobranchprimordium present at the anterior part close to the eyeball; (b) gill like structure of the pseudobranchprimordium; arrows indicate free lamellae, bc - blood cells, FA- filamental artery, GA - gill arch.

Erb, 1984). Interestingly the eel, *Anguilla anguilla* (Linnaeus, 1758) possesses a well-developed, uncovered pseudobranch at larval stage, whereas it is absent in adult fish (Laurent and Dunel-Erb, 1984). The type of pseudobranch with the free lamellae contains the chloride cells typical for the fish gills (Mattey *et al*., 1978; 1980; Sánchez-Amaya *et al*., 2007; Yang *et al*., 2014). Whereas in covered pseudobranchs, typical for the majority of freshwater fishes, chloride cells are absent (Mattey< *et al*., 1978; Fisher-Scherl and Hoffmann, 1986; Kern *et al*., 2002;

Waser *et al*., 2005; Hamidian and Alboghobeish, 2007). In the brackish water fishes, usually possessing the intermediate type of pseudobranchs, the chloride cells only occur on the lateral aspects of the pseudobranch (Mattey *et al*., 1978; King *et al*., 1993).

Primordial pseudobranch was composed of intensively dividing cells. Erythrocytes were also easy distinguishable (Fig. 5a, b). From 14 dphpseudobranch cells (typical for pseudobranch), pillar cells and epithelial cells were noted in the asp larvae pseudobranch. Lacunar tissue layers separated
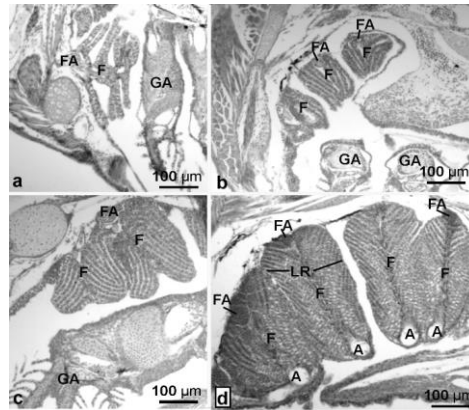
Figure 4. The *Leuciscusaspius* larvae pseudobranch; (a) with free lamellae at 9 dph; (b), (c), (d) as bud like structures at 14, 19 and 24 dph respectively; F - filament, FA- filamental artery, GA - gill arch, LR - lamellar region of one filament; (a), (c) – longitudinal-, (b), (d) – cross-section.
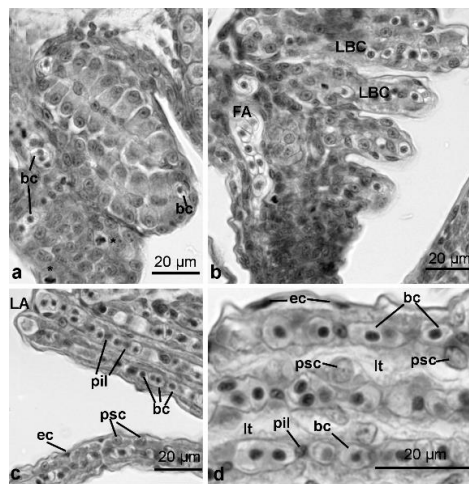

Figure 5. Histology of the pseudobranch lamellae during early ontogeny of *Leuciscusaspius* larvae; (a) asp pseudobranch at 4 dph - lamellar primordia composed of pseudobranchial cells, arrow indicates blood cells, asterisk (*) shows dividing cells; (b) asp pseudobranch at 4 dph - lamellae with blood capillaries; (c) asp pseudobranch at 14 dph with partially fused lamellae; (d) asp pseudobranch at 19 dph - completely fused lamellae; bc - blood cells, ec - epithelial cells, FA - filamental artery, LA - lamellar arterioles, LBC - lalmellar blood capillaries, lt - lacunar tissue, pil - pillar cells, psc - pseudobranchial cells; (a), (b), (d) -longitudinal-, (c) - cross-section.

the pseudobranch lamellae from the endothelium of capillaries (Fig. 5c, d). Neither chloride cells nor mucus cells were observedin asp and *D. dentex* larvae pseudobranchs till the end of study (24 and 36 dph respectively, exotrophic stage started at 9 dph and 10 dph respectively) (present study; Santamaría *et al*., 2004). Sánchez-Amaya *et al*. (2007) observed the chloride cells in *P. auriga*pseudobranch larvae from 11-12 dph (the beginning of the exotrophic stage) and mucus cells were noted from 29 dph, after differentiation of the gastric glands in the stomach.

In this study the morphometric and histological analyses revealed that the morphology of asp pseudobranch changes when larvae become older and larger. The pseudobranch of *Leuciscusaspius* larvae evolved from type I in the Bertin's (1958) classication by the end of endo-exotrophic stage to type IIB some days after the beginning of exotrophic stage (by 14 dph). The similar situation was described in *Cyprinuscarpio* Linnaeus, 1758 and Atlantic code (Harder, 1975; Morrison, 1993). We considered that in fishes possessing covered and embedded pseudobranch the early development of this organ occurs in similar way, starting from the free-lamellar type. In these fishes the chloride cells of the pseudobranch are probably absent independently of their freshwater or saltwater habitat.

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