Reproductive isolation between two sympatric species from genus *Rutilus* from Lake Skadar (Montenegro)

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Abstract
Lake Skadar basin is inhabited by two species from genus Rutilus: *R. prespensis* (Karaman, 1924) and *R. albus* Marić, 2010. These species have never been reported to hybridise in nature. Seasonal isolation is developed, since there is no existing overlap in time of spawning. In this study, histological features oocyte developmental stages were described in detail using light microscopy. The *R. prespensis* and *R. albus* have a group-synchronous ovary, spawn once a year and have a relatively short breeding season. *R. albus* spawns in February, while *R. prespensis* spawns in April-May. The combination of different spawning times and places are considered to be powerful barriers to hybridization.

Keywords: Spawning, endemic, *zona radiate*, *Rutilus albus*, *Rutilus prespensis*.

Introduction

Lake Skadar is the largest Balkan lake, a region well recognized as a European biodiversity hotspot (Gaston & David 1994; Griffiths *et al*., 2004) and one of the key biodiversity areas in the Mediterranean (Darwall *et al*., 2014). Together with Lake Prespa and Ohrid, it is classified as part of a group of ancient Balkan lakes (Glöer & Pešić, 2007). For those reasons, it is invaluable to research every biological aspect of both endemic species and those with a wider range of distribution.

The existence of two species from the genus *Rutilus*, in Lake Skadar, was first suggested by Marić, in 1989, who describes two subspecies: *R basak ohridanus* and *R prespensis vukovici*. In 2011, Milošević *et al*., showed that the Lake Skadar basin is, in fact, inhabited by two species from genus Rutilus: *R. prespensis* (Karaman, 1924) (local name yellow roach) and *R. albus* Marić, 2010 (white roach). The species from the genus Rutilus are one of the most numerous fish species in Lake Skadar, especially *R. prespensis*. *R. albus* is an endemic species for Lake Skadar. Recent studies of these species have been related to taxonomic status and length-weight relationships (Marić & Radujković, 2009; Milošević *et al*., 2011; Milošević *et al*., 2012). Until now, no research was undertaken to describe their adaptation to a life of sympatry. As reproductive isolation is a precondition for sympatry and as reproduction is regarded as a key mechanism for the survival of a given species

(generally, fish species are characterised by a specific type of ovary organisation, fertility and spawning) (McMillan, 2007), an analysis of the ovarian cycle of two sympatric species would pave the way for further studies involving sympatry and associated adaptations. Therefore, this study aimed to obtain information about temporal and spatial differentiation of sympatric *R. prespensis* and *R. albus* by analysing the ovarian cycle. In addition, this study also provides the first available insight regarding the reproduction mechanism about these sympatric taxa.

Material and methods

A total of 40 mature female of *R. prespensis* and *R. albus* from Lake Skadar were collected from October to May using 14-16 mm mesh-sized sinking gill nets and electro-aggregates, depending on the place of sampling. The specimens were sampled from several different, primary littoral sites. A visual determination of maturity was made based on external appearance of body, colour, gonad size and weight, blood vessel distribution and the degree of the oocyte maturity level. The level of sexual maturity, based on the external appearance of gonads, was determined using a scale according to Nikolsky (1963). To prepare the histological sections, middle segment of the left ovary was fixed in buffer formalin, dehydrated through a series of increasing concentrations of ethanol, cleared with xylene and finally embedded in paraffin. Sections of 5-6 μm were stained with standard hematoxylin-eosin procedure (H&E). Mounted slides were examined using binocular light microscopy. In order to analyse the ovarian cycle, the developmental stages of studied oocytes were divided into three main phases: I Previtellogenic phase (primary oocyte and perinucleolus stages); II Vitellogenic (Cortical alveolus and vitellogenic stages) and III Maturation (Early and final maturation stages) (Heidari *et al*., 2009).

Results

I Analysis of reproductive cycle of the *R. albus*

Rutilus albus has a group-synchronous ovary and spawn once a year on pebbly bottoms at a water temperature ≤10°C. Based on morphological features (macroscopic analysis of gonads) and histological observations we obtained the following results:

1. November. The ovaries exhibited an intense yellow colour, occupying a large portion of the abdominal cavity. Blood vessels were clearly noticeable. They were found to be in the IIIrd stage of maturity. The oocytes could separate from each other. According to histological observations oocyte were found to be in the vittelogenic phase (Figure 1a). Several nucleoli appear at the periphery of the nucleus. The appearance of the cortical vesicle, was observed to be spherical on the periphery of the cytoplasm. The number and size of yolk vesicles increased. *Zona radiate*, with 15μm thickness was observed.
2. January. The ovaries exhibited an amber colour, occupying most of the abdominal cavity. They were found to be in the IVth stage of maturity, indicating this as the period prior to spawning. A histological analysis determined that oocytes were in the maturation phase (Figure 1b). They were also filled with yolk. *Zona radiate* thickness measurements ranged between 20 and 25 μm.

3. February. Spawning commenced at the end of February. Gonads were found to be in the Vth stage of maturity. During this period, the sex of the individual fish can be established based on their phenotypic distinctions. The males exhibited tubercles almost all over their body. The most expressed tubercles were found to be on the dorsal side of both the head and the abdominal part of the body, including the dorsal and anal fin. A light touch of the male's abdomen produces an outflow of sperm. Histological analysis also confirms that spawning was completed (Figure 1c). A large number of empty follicles were observed. A regeneration of the germinal epithelium was observed as well as a few atretic follicles in the degeneration and resorption phase (Figure 1d).
4. April. The gonads were found to be in the regeneration phase (VI stage). The ovaries were a grey colour, with dark specks interspersed, indicating the process of reabsorption of mature, unrejected oocytes has commenced. Histological analysis indicates the presence of post ovulation follicles (Figure 1e), individual atretic follicles and oocytes in the perinuclear phase (Figure 1f). The presence of oocytes in the previtellogenic phase would suggest that the process of oocyte maturation has begun. This type of gonad state is suggestive of a change from stage VI to stage II of maturity.

II Analysis of reproductive cycle of the *R. prespensis*

R. prespensis has a group-synchronous ovary and spawns once a year on macrophyte vegetation at a water temperature of 15°C. Based on macroscopic analysis of gonads and histological observations we get following results:

1. November. The ovaries were a bright yellow colour with an easily identifiable blood vessel. The egg granules were easily observable and in the maturing phase. Histological observations indicate that oocytes are in the vitellogenic phase, cortical alveolar phase (Figure 2a). The cytoplasm is almost completely filled with cortical alveoli; it is only alongside the outer edge of the nuclear membrane that the commencement of yolk vesicles accumulation was observed. *Zona radiate* thickness was measured to be 7.5 µm.
2. December – January. The ovaries are in the IVth stage of maturity. Histological analysis suggested that the oocytes were in the vitellogenic phase (Figure 2b). Around the nucleus, an intensive grouping of yolk vesicles was observed, with a tendency for peripheral expansion. However, the peripheral part of the cytoplasm remained filled with cortical alveoli. *Zona radiate* thickness was measured to be between 12.5 to 15 µm.
3. February. The ovaries are in the IVth stage of maturity. They occupied a large portion of the abdominal cavity. They were an intense yellow colour with easily separated egg granules. Histological analysis indicated that oocytes are in the maturation phase (Figure 2c). Egg vesicles occupied a large portion of the cytoplasm. *Zona radiate* thickness ranged from 15 to 20 µm.
4. April. The ovaries are in the Vth stage of maturity. During this period, the sex of the individual fish can be established based on their phenotypic distinctions. Males exhibited tubercles all over their body. The most noticeable tubercles were found on the operculum, dorsal side of the body and the dorsal and anal fin. Red spots were also found on the operculum and fins. A microscopic analysis showed that oocytes are completely morphologically differentiated and that they reached the final maturation phase (Figure 2d). The yolk content completely filled the oocyte. The spawning period was established to be the end of April and during May.

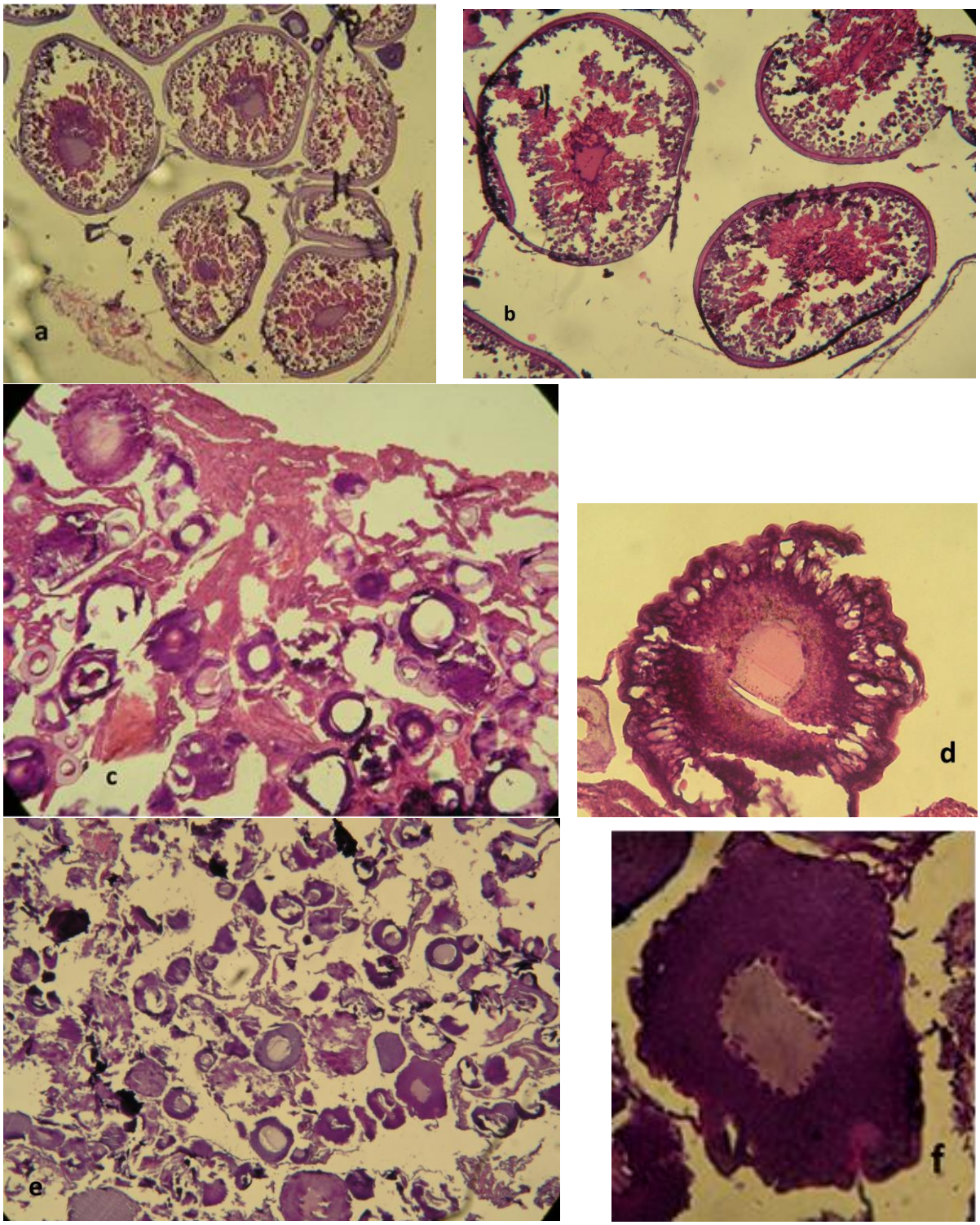

Figure 1. Cross-sections of ovaries of *R. albus*. a) Oocyte in vitellogenic stage. Cortical alveoli are progressively displaced towards periphery while number and size of yolk vesicle increased. 5x H&E; b) Oocyte in maturation stage. Vesicle in oocyte plasma form bigger vesicle by integration. 5x H&E; c) Ovum after completed spawning. Empty follicles and the regeneration of the germinal epithelium was observed. 10x H&E; d) Atretic follicles 20x H&E; e) Post ovulation follicles and oocytes being readied for future spawning. 10x H&E; f) Perinuclear stage of primary oocyte growth. Ooplasma was intensely stained while the nucleus was not. Several nucleoli appear at the periphery of nucleus. 40x H&E.

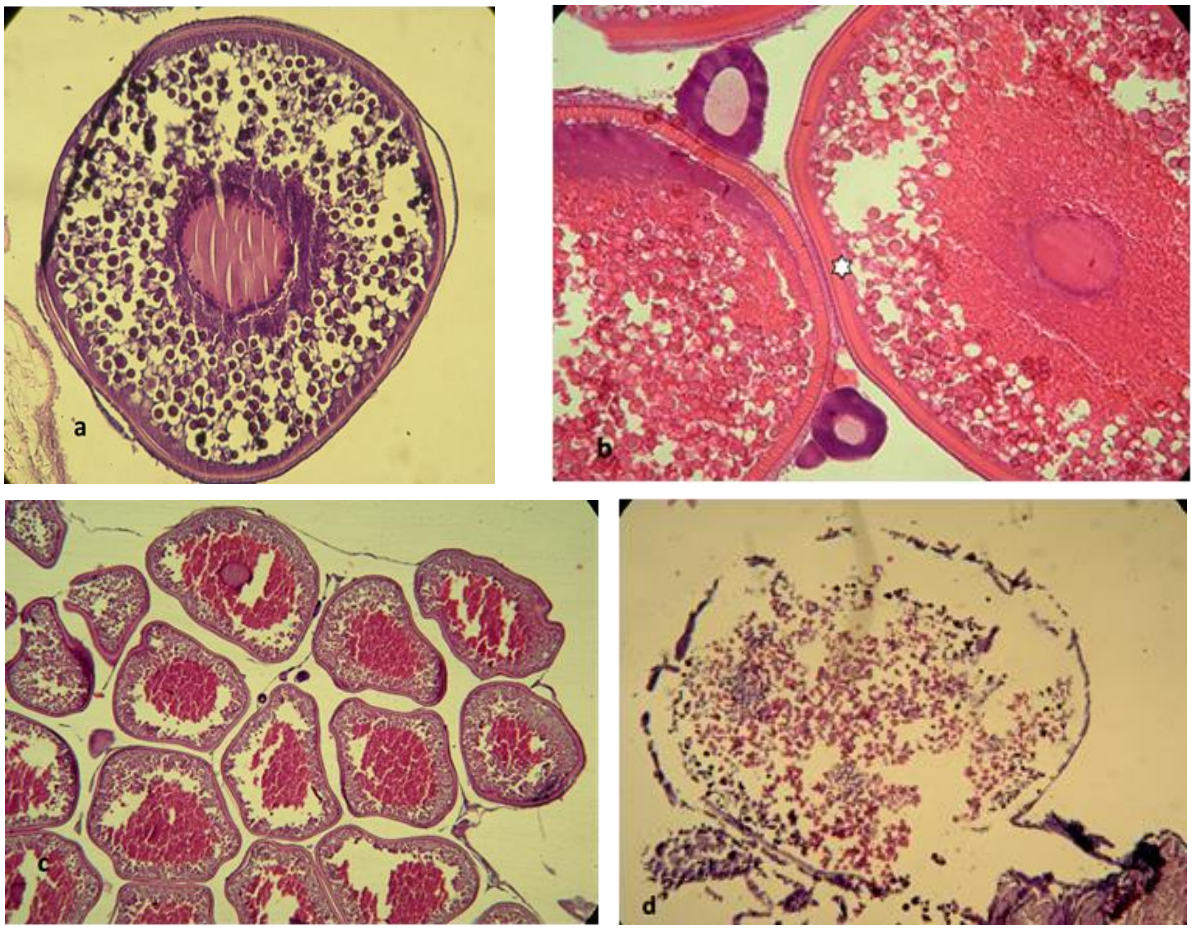

Figure 2. Cross-sections of ovaries of *R. prespensis*. a) Oocyte in cortical alveolar stage. Cortical alveoli fill the oocyte cytoplasm. 20x H&E; b) Oocyte in vitellogenic stage. Yolk vesicles grouping around the nucleus. Clearly visible *zona radiate* (star marked) Oocytes in the primary growth phase. 5x H&E; c) Oocyte in maturation stage. 10x H&E; d) Oocyte in final maturation stage. Yolk material completely fills the inside of the oocyte. 10x H&E.

Discussion

Considering the established morphological and genetic differentiation of sympatric taxa from Lake Skadar (Milošević *et al.,* 2011) it was necessary to analyse the sympatric taxa reproductive cycle in order to determine their adaptability to a life of sympatry. Marić, 2010, was the only available study regarding the reproduction of sympatric species from Lake Skadar. In this study, Marić provided evidence suggesting that *R. albus* spawns earlier in the season than *R. ohridanus* and that a sexually mature *R. albus* female (TL = 220.1 mm, SL = 181.0 mm), caught on the 15 January, was in the IVth stage of maturity.

A lack of sexual dimorphism, with regard to the members of the genus Rutilus, was highlighted by Stangenberg, 1947; Baranyi *et al*., 1997. This study showed that there is no phenotypic differentiation between the sexes in individuals from either taxa prior to spawning. During spawning, males began to develop tubercles. *R. albus* tubercles were fist observed in February and *R. prespensis* in April. *R. prespensis* males

also exhibited red spots on the operculum and fins. The existence of fine tubercles is one of the characteristics of the genus Rutilus, however, Bogutskaya & Iliadou (2006) have highlighted that some species of this genus lack them (although they do not specify which species).

R. albus and *R. prespensis* oocyte development stages are in accordance with other teleostes like *Danio rerio* (Maack & Segner, 2003), *Cyprinus carpio* (Gupta, 1975), *Rutilus frisii* (Heidari *et al*., 2009). The *R. prespensis* and *R. albus* have a group-synchronous ovary, spawns once a year and have relatively short breeding seasons. These results are in accordance with Paykan-Heyrati *et al*., 2007 and Heidari *et al*., 2009. However, these results are in stark contrast with the only documented genus *Rutilus* results from Lake Skadar. Knežević and Ivanović (1975) documented an asynchronous pattern or growth of oocytes and partial spawning for *R. rubilio* from Lake Skadar. The same authors suggest that the spawning period of this species is from March through to June, with mass spawning observed in April and May. In this instance the authors, Knežević & Ivanović (1975), recorded a spawning period of four months which far exceeds current studies involving spawning patterns. The most probable explanation for this unlikely spawning period is that the authors of this study, at the time, were unaware that they were using fish of not one species but rather, two sympatric species.

The results from our study indicated that differences between the examined species manifested themselves in reaching certain developmental stages at different times, including the spawning period. Namely, *R. albus* was consistently detected to be in a more advanced maturation phase than *R. prespensis* at any given time. *R. albus* spawns in February, while *R. prespensis* spawns in April-May. Readying for spawning and the spawning timeframe coincide with the monthly variation of the conditioning factor (Milošević *et al*., 2012). From the established spawning periods, a conclusion can be reached that the sympatric taxa differ with regard to environmental factors, mainly temperature, one of the main factors affecting spawning. Considering that *R. albus* spawning occurred at the end of February it can be concluded that this species, with regard to environmental demands, is similar to *Chondrostoma ohridanum* Karaman, S., 1924. It spawns during a winter period (February through to March) at a water temperature of 8 to 10°C (Ivanović & Knezević, 1978). The result of presented study showed that the *Zona radiate* begins to form in the cortical alveolar stage, with maximum dimensions reached during the mature stage. This conclusion is in line with Heidari *et al.,* 2009. Considering that in the egg cells of studied species, the presence of oil drops was not observed, the eggs of studied species can be classified under the demersal egg group (Heidari *et al.*, 2009). This interpretation is supported by the observed thickness of the *Zona radiate* layer of both taxa, *R. albus* 20-25 µm, and *R. prespensis* 20 µm. These results are similar to those observed in *R. frissi* where the *Zona radiate dimensions* were 19,8±1,4µm (Heidari et al., 2009). *R. frissi* deposits it's eggs on gravel or macrophyte covered substrates (Heidari et al., 2009). The thickness of this layer was observed to be different in different teleost species. For example, *Zona radiate* dimensions were between 5 to 9 µm for *Donio rerio* (Çakici&Üçüncü, 2007) and 7-8 µm for *Carrasius auratus* and up to 30 µm for the *Salmo gairdneri* (Nagahama, 1983). The existence of differences in this acellular layer would also point towards differences in ecological conditions and would explain the development of a thicker chorion in demersal eggs (Suzuki *et al.*, 2000). The ticker chorion can provide a mechanic protection against abrasion of the bottom, as noted in *Salmo gairdneri* (Nagahama, 1983). During the spawning period, *R. albus* swims to inflows, which leads to the conclusion that egg deposition occurs on sandy and gravel like

substrate. The thickness of the *Zona radiate* also suggests that fact, taking into consideration that a phytophilic fish species such as *C. auratus*, which deposits eggs on macrophyte covered substrates, has a *Zona radiate* thickness of 7 to 8 μm, whereas the genus *Salmo,* which deposits demersal (lithophilic) eggs on gravel like and rocky substrate, have a *Zona radiate* thickness of 30 μm. According to Marić, 2010, *R. albus* average egg size was around 1.8 mm and around 470 units of mature eggs per 1 gram was counted.

R.albus and *R. prespensis* also differ with regard to the time it takes to develop sexual maturity. Namely, *R. albus* develop sexual maturity in the third year whereas *R. prespensis* do so in the second year. In addition, these two-sympatric species develop sexual maturity at different lengths. For example, at lengths between 12.9 to 13.7, *R. albus* fish are sexually immature, whereas *R. prespensis* have already reached sexual maturity at these lengths (unpublished data). These results are in accordance with Marić, 2010, whose study showed that *R. albus* specimens (n = 60) up to 120 mm in length (SL) and 35 g in weight, caught in February were sexually immature.

Understanding the key periods of an endemic (and otherwise) species life cycle is relevant not only from a scientific but also from a practical perspective. Studying and analysing the process of reproduction, the development of sexual maturity, spawning habitats and timeframes, always assist in not only determining the condition of a population but also its level of vulnerability. In this paper, we present evidence of spatial and temporal reproductive isolation of a sympatric species, *R. albus* and *R. prespensis* which is a powerful barrier to hybridization. Also, the findings of this paper present first comprehensive data about reproductive biology and ovarian cycles of species from genus Rutilus from the ancient Balkan lakes.

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