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Female reproductive cycle of the Dusky grouper, *Epinephelus marginatus* (LOWE, 1834) in captivity

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The paper shows the characteristics of oogenesis in the dusky grouper, Epinephelus marginatus *(LOWE, 1834) under conditions of captivity in the south-eastern Adriatic. We found no visible ovarian cytological changes or oocytes growth during annual reproductive cycle from October to May. Oocytes started to develop and grow at the beginning of June, when ambient sea temperatures surpass 20 °C. The process of vitellogenesis lasts three months, during which time the oocytes grow from 80 to 500 μm in diameter, reaching the size needed for inducing hormonal spawning. Three groups of cells are present in the ovaries: oogonia, previtellogenic oocytes and oocytes in the process of vitellogenesis. During these three months, there is an ongoing process of where new oocytes develop from primary oogonia and grow up to 20-50 μm, as well as the growth of groups of previtellogenic oocytes up to 50-90 μm, along with the development of a number of morphological changes, of which the most prominent is the fragmentation of nucleoli and their migration towards the periphery of the nucleus.*

We observed a significant percentage of oocyte atresia in our broodstock and found it to be a main problem in the artificial spawning.

Key words: Dusky grouper, *Epinephelus marginatus*, oogenesis, oocyte, development, temperature, atresia

INTRODUCTION

The dusky grouper, *Epinephelus marginatus* (LOWE, 1834) is a highly valued fish in the Mediterranean. Because of overexploitation, it became very rare in some countries (ZABALA *et al.*, 1997). In the last few years, due to a promising growth rate (GRACIA LOPEZ and CASTELLO-ORVAY, 1995) and a good market price (15 US $), the species is of interest for aquaculture and restocking purposes.

The main problem for the successful propagation of this fish in the Mediterranean is its protogynous hermaphroditism. It matures as a female at 3 kg of weight, and changes sex to male later, usually when above 10 kg in weight (JARDAS, 1996). The problem of larger males in reproduction studies was overcome with the successful sex reversal of smaller females (0.15-3 kg of weight), by adding 17α methyltestosterone to the food (GLAMUZINA *et al.*, 1998a). However, the artificial spawning of females using different hormones still presents

serious problems. Although the successful spawning (SPEDICATO *et al.*, 1995; GLAMUZINA, 1998), first larval rearing trial (GLAMUZINA *et al.*, 1998b), egg and larval characteristics (GLAMUZINA *et al.*, 1998c) have been described, all papers reported problems in the establishing of an appropriate hormone protocol for successful spawning.

A primary requirement in aquaculture propagation is the ability to fully control the sexual maturation and spawning of the species under cultivation (BROMAGE, 1995). The main problem in establishing this control is the lack of a comprehensive knowledge of the female sexual cycle. Once described and well understood, oogenesis has led to an ability to control reproduction in many species.

This paper describes characteristics of oogenesis during the annual reproductive cycle of the dusky grouper, *Epinephelus marginatus* held in captivity in the south-eastern Adriatic region.

MATERIAL AND METHODS

Oogenesis was observed using 15 females, with weight ranging from 1.5 to 13 kg, and age from 3 to 14 years. The fish were kept in facilities of Dubrovnik public aquaria in 10 m^3 concrete tanks with added stones and flow-through of ambient seawater (temperature ranged from 13-24° C and salinity from 37-38 psu) and natural photoperiods. The temperature values for 1996,1997 and 1998 are presented in Fig.1.

The fish were fed to satiation three times per week with trash fish, mainly sardines.

During 1996, 1997 and 1998, the fish were examined once monthly. After anaesthetization in benzocain, ovarian samples were taken by biopsy with the insertion of a plastic tube (inner diameter 1 mm) into the oviduct and sucking a sample of ovarian material into the tube. The gonad tissue was immediately examined under a light microscope in order to determine oocyte type and dimension. Fifty oocytes of each type found in the samples were measured and average diameter calculated. One hundred oocytes of different types in one microscope field were measured in each sample to establish their frequency distribution during the annual reproduction cycle. Remaining tissue was preserved in 10 % formaldehyde. Permanent preparations were made based on this tissue. Histological sections were cut with a microtome and the ovarian tissue sections stained with eosin and hematoxylin. Photographs of these permanent preparations were made using the camera attached on light microscope.

Each year during August, one group of fish was treated with HCG or LHRH $_{(a)}$ hormones and the other group injected with distilled water only. Oocyte growth after those treatments was observed. Details of hormonal treatments were described earlier (GLAMUZINA *et al.*, 1998b).

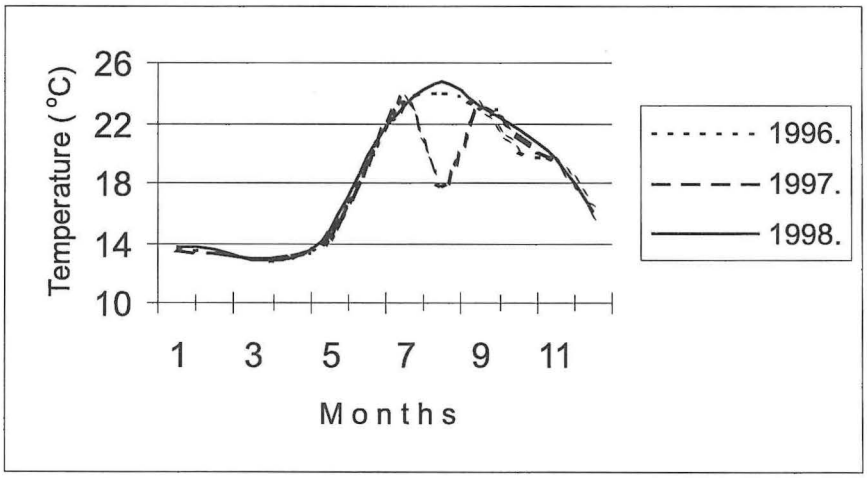


Fig. 1. Ambient sea water temperatures during brood stock conditioning of dusky grouper, Epinephelus marginatus *in 1996, 1997 and 1998 year*

RESULTS

In the period from the beginning of October to the beginning of May, (autumn, winter and spring) three types of oocytes of small dimensions were seen in the ovaries of the dusky grouper. These oocytes were: group of oocytes, averaging 67 ± 18 μm (mean diameter standard deviation), that had a nucleus distinctly separated from the cytoplasm, but without any other visible changes; a group of oocytes, averaging 32 ± 19 μm in diameter, remained without any visible difference between the nucleus and the cytoplasm; and a third group of cells represented by a group of oogonia, sized about 10 μm in diameter and found throughout the entire ovary sample (Fig. 2a).

A growth of oocytes was seen in mid-June, especially with the first group of larger oocytes averaging from 98 ± 29 μm. Here, differentiation of the nucleus and the formation of more nucleoli migrating from the centre to the peripheral regions had already started. Oocytes in which vitellogenesis had just started were present, as well as those were it was well advanced (Figs. 2b,c). Alongside this group, there were smaller oocytes sized around 35 μm in the ovaries, as in April, which were uniformly stained.

By mid-July, the size of oocytes, which had started the process of vitellogenesis, was 250 ± 85 μm (with sizes varying from 150-380 μm). Yolk and oil droplets were present in the cytoplasm, while the nucleus still took a central position in the cell. The second group of oocytes had grown to 45 ± 15 μm in diameter, but did not change their cytological appearance, and continued to remain in a basophilic phase. The ovaries continued to show large formations of oogonia (Fig. 2d).

By mid-August, the average size of vitellogenic oocytes was 499 ± 172 μm. The cytoplasm clearly showed yolk and oil droplets which started to coalesce. The nucleus was no longer visible within the centre of the oocyte, rather, it had migrated towards the periphery. In August, two distinct groups of previtellogenic oocytes were visible in the gonad, the larger (mean diameter 55 ± 18 μm) with a clearly separate nucleus and cytoplasm, and the smaller (mean diameter 25 ± 9 μm) uniformly stained.

A hormone treatment was started in August. With the first injection, there was a slight increase in the size of oocytes from 500 μm to 600-650 μm, with clearly recognizable structural changes. The yolk droplets lost their shape, whereby the cytoplasm changed from the granular phase, and the egg cells became all the more cleaner, losing their colour and becoming transparent (Fig. 2e).

Following the second hormone injection, the size of oocytes rapidly increased to 800 μm within the next 10-20 hours. Due to structural changes, the oil droplets united into one or more larger oil drops. In the group of fish treated with blank solution, the oocytes remained the same as in August and afterwards started a process overrippening and resorption.

Following spawning, during September, the ovaries showed large vitellogenic oocytes which had not spawned and which basically were in the process of resorption.

The majority of stained oocytes were sized between 30-90 μm, of which those over 50 μm had an unstained nucleus. These samples also showed large groupings of primary oogonia (Fig. 2f).

Changes in oocyte size are correlated with increases in ambient sea temperatures. Significant changes in the growth of certain groups of oocytes during three years of investigations were noticed only at temperatures over 20 °C, or in the period from June to August (Fig. 3). However, we observed slower oocytes growth during 1997, which correspond with lowering of seawater temperatures during July and August (Fig.1). So, the oocyte growth from this year is not included in Fig. 3.

During 1996, 1998 and 1999 only few females showed normal cycle of oocytes development after hormonal treatments. We observed a significant number of females (six in 1996, seven in 1998 and five in 1999) which undergone oocyte atresia and their fast resorption. For example: after hormonal treatments in 1996 we stripped two biggest females (13 and 12 kilos)

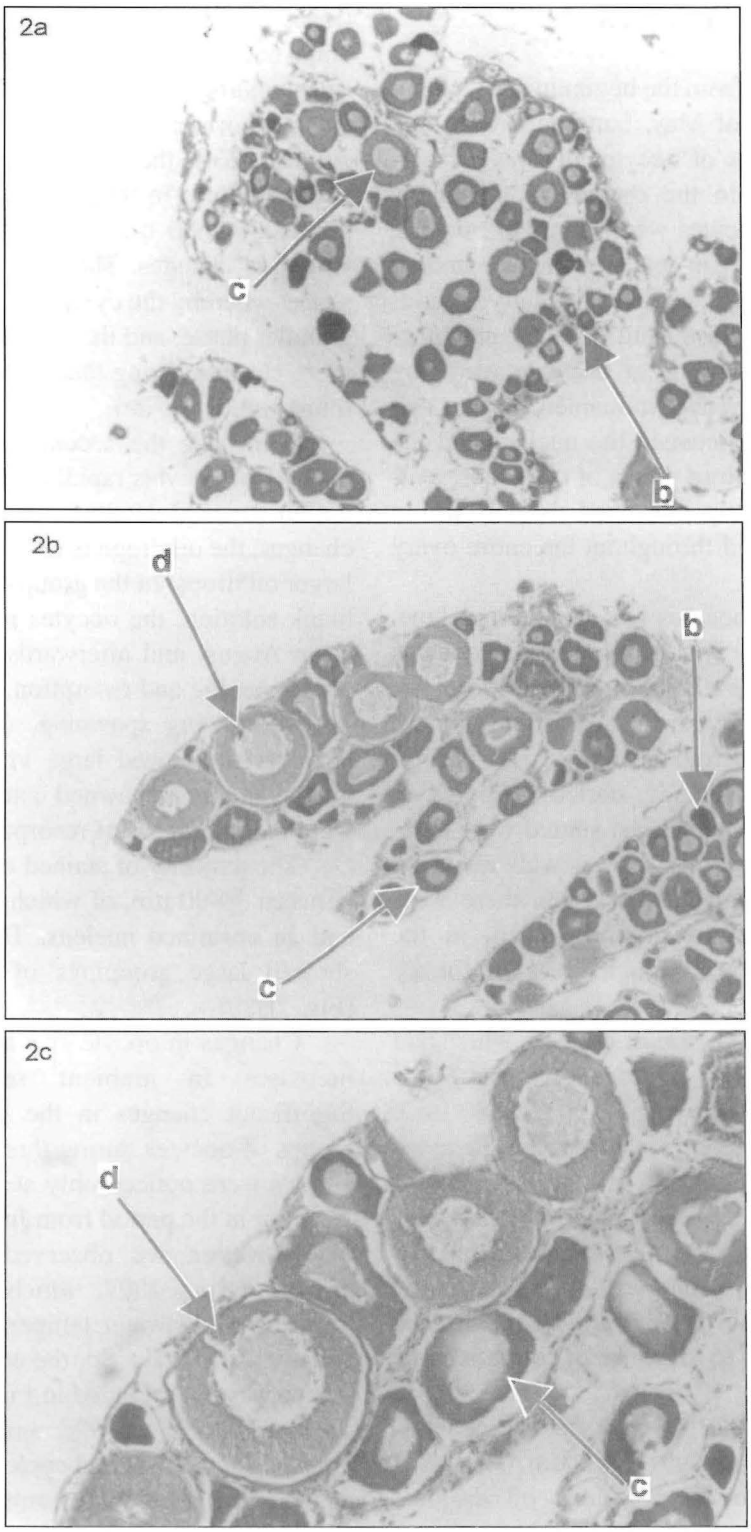
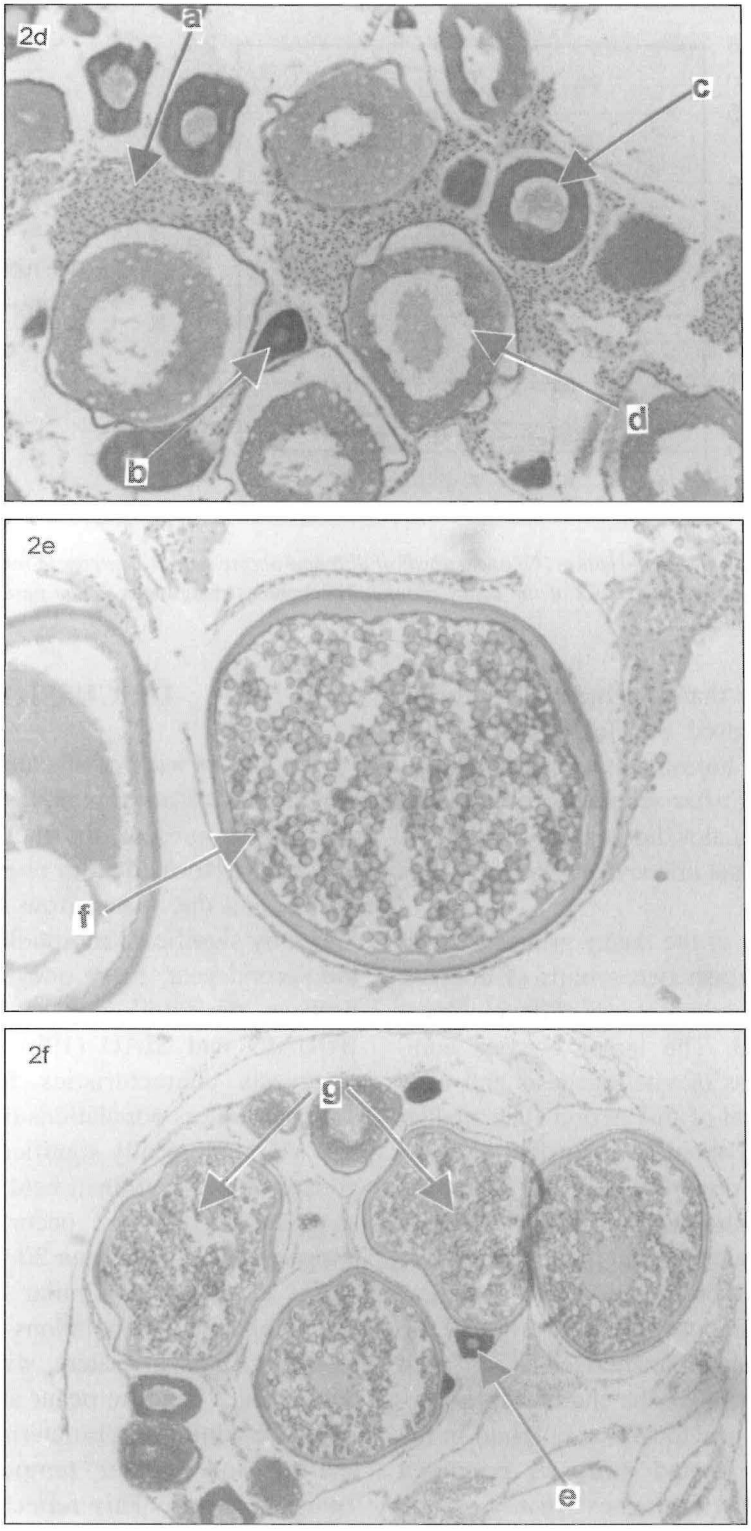


Fig. 2. Ovaries of the dusky grouper, Epinephelus marginatus *held in captivity under ambiental south-eastern Adriatic conditions:*
2a- October-May (magnification x 100);
2b- June (x100);
2c- June (x160);



2d- July (x160);
2e-August (x100);
2f-September (x160)
(a-oogonia; b- smaller previtellogenic oocytes; c- bigger previtellogenic oocytes; d- early vitellogenic oocytes; e- newly recruited oocyte; f- mature vitellogenic oocyte; g- atretic oocytes)

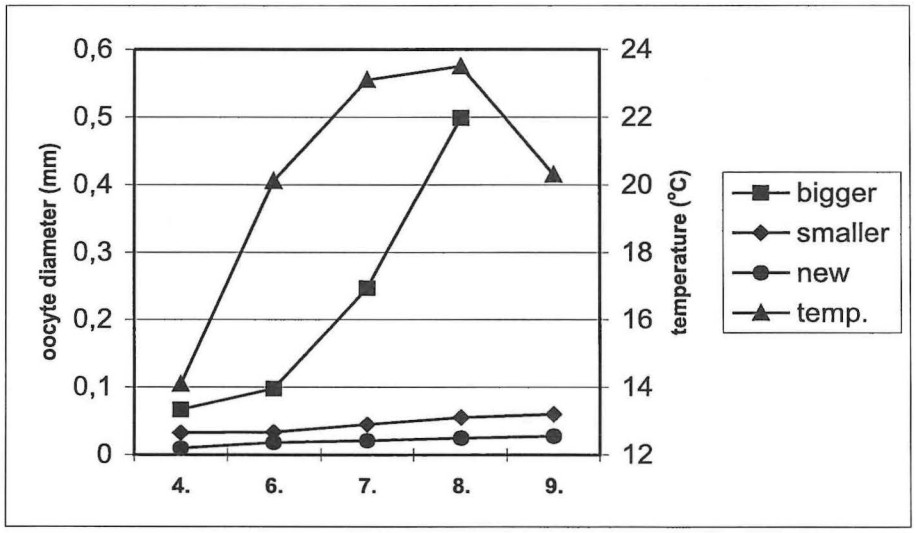


Fig. 3. Seawater temperature (C) and growth of different oocyte types (diameter in mm) during intensive oogenesis of the dusky grouper Epinephelus marginatus *in the period April-September*

and obtained more than two litres of fluid consisted of decomposed oocytes. During 1997, only one of ten hormonally treated females spawned, but only after second hormone treatment. All other females did not respond and we found signs of atresia in oocytes of only 500 μm size.

In the ovaries of the dusky grouper, it was possible to distinguish two groups of oocytes, which underwent various stages of development during this period. The larger oocytes commenced the process of vitellogenesis and were spawned by the end of this period. The smaller oocytes grew from about 30 m to sizes between 50-90 μm, and covered various structural changes of which the most prominent were the fragmentation of nucleoli and their migration to nucleus periphery. From the oogonia, a new generation of smaller oocytes sized from 30 μm upwards was formed, characterised by uniformly colored cytoplasm. By the end of September, oocytes described previously were found in the gonads, and this gonad structure remained unchanged until May of the next year.

No differences were found in ovary structure or in the speed of oogenesis, in relation to female size. All females between 3 and 13 kg had similar ovarian structure during annual reproductive cycle.

DISCUSSION

In the ovaries of the dusky grouper, two generations of oocytes and groups of oogonia are always present. In the first year, some oocytes are formed from oogonia and grow to 30 μm and the others grow to 50-90 μm, followed by significant morphological changes. In the second year, these oocytes commence the process of vitellogenesis to mature egg. BOUAIN and SIAU (1983) describe similar oogenesis characteristics for wild fish in Tunisian waters populations of dusky grouper.

Morphologically significant changes in the gonads of dusky grouper held in captivity in the south-eastern Adriatic occur only at ambient temperatures greater than 20 °C. BOUAIN and SIAU (1983) give similar results in gonad development for populations of dusky grouper found in Tunisian waters, with a difference in that changes begin to occur at the beginning of April. The difference is determined by the faster heating and greater temperature totals of Tunisian waters. This reflects on the start of spawning, which starts in June in Tunisian waters (BOUAIN and SIAU, 1983), and at the end of August in the waters of the south-eastern Adriatic (SKARAMUCA *et al.*, 1989). It can be indirectly concluded that temperature has a

major role in the process of gonad maturation throughout the whole year. Seems that photo-period do not have any role in dusky grouper oogenesis, because the negative effect of prolonged photo-period (16L-8D) on oocyte development was reported earlier (GLAMUZINA, 1998). This could be explained with living habits of this fish which spends much of their life cycle in stone caves (JARDAS, 1996). The fact that oocyte growth is not observed at temperatures below 20° C points towards the tropical origin of the species. Tropical characteristics are significantly limited in the border regions of species geographical distribution, as is the case with populations in the south-eastern Adriatic. All research in this region has established problems with artificial spawning using hormone treatments, so that a successful protocol has not yet been developed (SPEDICATO *et al.*, 1995; GLAMUZINA *et al.*, 1998b). One of the causes can possibly be the lack of total temperatures required for the successful completion of oogenesis, as both research mentioned were carried out under similar ambient conditions. This is demonstrated by the temporary absence of new generations of young grouper noted in the south-eastern Adriatic, which might be indicative of the absence of natural spawning during colder years (SKARAMUCA, unpublished data). One such year was 1997, when during July and August the water temperatures of the south-eastern Adriatic dropped from 23° C to only 18° C, which lasted up to twenty days (Fig.1). Attempts at hormonal treatments during this year gave very poor results (GLAMUZINA, 1998).

We observed a significant percent (from 60-75 %) of atretic females each year during our research. The incidence of atresia is increased under suboptimal conditions and is a result of environmental or other stress (TYLER and SUMPTER, 1996), reduced feeding and starvation (BROMAGE and CUMARANATUNGA, 1988) or handling stress (WALLACE *et al.*, 1993). Most of these factors are present in our working schedule and is difficult to chose which one is responsible. However, the fact that occurrence of atresia is a result of suboptimal conditions, points to the conclusion that our brood stock needs a better conditioning and feeding.

From the characteristics of oogenesis in the dusky grouper held in captivity in south-eastern Adriatic and Tunisian water conditions, as well as from the numerous published papers on the spontaneous spawning of related tropical species over a period of a few months (TUCKER *et al.*, 1996; JAMES *et al.*, 1997), various possibilities arise from temperature manipulation so that the dusky grouper can successfully spawn at the required time. Future research will determine the possibility of promoting maturation and artificial spawning towards the beginning of summer, in order to ensure high summer temperatures for the rearing of larvae and fingerlings.

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Reproduktivni ciklus ženki kirnje goleme, *Epinephelus marginatus* (LOWE, 1834) u zatočeništvu

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SAŽETAK

U radu su izložene značajke oogeneze kirnje goleme, *Epinephelus marginatus* (LOWE, 1834) u uvjetima zatočeništva u jugoistočnom Jadranu. Tijekom godišnjeg reproduktivnog ciklusa od rujna do svibnja nisu utvrđene značajne citološke promjene ovarija ili rast oocita. Razvitak oocita započima početkom lipnja, kada ambijentalna temperatura morske vode prijeđe razinu od 20° C. Proces vitelogeneze traje tri mjeseca, tijekom kojih oociti narastu od 80 do 500 μm, dostižući veličinu pogodnu za uspješno umjetno hormonalno mriješćenje. U ovarijima se tijekom razdoblja lipanj-kolovoz nalaze tri grupe jajnih stanica: oogonije, previtelusni i vitelusni oociti. Tijekom ovog razdoblja stalno je i novačenje novih oocita koji narastu od 20 do 50 μm, kao i rast previtelusnih oocita do 50-90 μm. Najvidljivija citološka promjena je fragmentacija nukleolusa i njegova migracija prema periferiji jezgre. Kod velikog broja riba u matičnom stoku uočen je značajan postotak degeneracije oocita, te ovu pojavu smatramo glavnim razlogom loših rezultata umjetnog mriješćenja.