

Original paper

The influence of the planting time and conditions on the reproductive properties of *Hirudo verbana* and *Hirudo medicinalis*

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ABSTRACT. Factors that affect on the sexual behavior of leeches not only in nature, but also in artificial conditions remain largely unexplored. We did not find data on the behavior of medicinal leeches, which are not placed in time from the aquatic environment in the peat-soil conditions after the appearance of clitella on their bodies. Therefore, the study of this problem has become relevant. For the study, four experimental animals groups were formed: 1 control – medicinal leeches (*H. verbana*), which were allowed to breed immediately after the appearance of clitella; 2 experimental – medicinal leeches (*H. verbana*), which were kept in an aqueous medium after the appearance of clitella for a month and then allowed to breed; 3 control – medicinal leeches (*H. medicinalis*), which were allowed to breed immediately after the appearance of clitella; 4 experimental – medicinal leeches (*H. medicinalis*), which were kept in an aqueous medium after the appearance of fertilization belts for a month then allowed to breed. The 400 sexually mature healthy medical leeches of two types aged 1.5–2 years were used in experiments. Initially, four individuals were placed into 4-liter containers for sexual intercourse, and after the appearance of differentiated fertilization zones, the control groups were immediately allowed to breed in a peat-soil environment. The experimental groups were left in the same containers for a month and then the survived leeches were allowed to breed. As a result of our research, we found mortality in the experimental groups. From the first week, we found that mortality before breeding of *H. verbana* was $15.6 \pm 2.1\%$ and of *H. medicinalis* – $14.3 \pm 1.5\%$. Also, $92 \pm 0.9\%$ leeches from experimental group died in pet-soil environment sometimes even without cocoon deposition. The experimental group had defective cocoons, but mostly dead offspring.

Keywords: leeches, reproduction, clitella, reproductive system

Introduction

As a result of the significant decrease of medicinal leeches in nature [1], more and more scientists around the world are looking for ways to increase their survival and reproduction under artificial conditions, with the maintenance of abiotic factors: water regime, temperature, light mode, mineralization, pH of water, oxygen concentration in water, etc. [2,3]. For example, studies [3] showed that in artificial conditions, preliminary isolation of leeches from each other and changing water and increasing temperature, also increases the process of copulation and survival. So, under natural conditions, leeches show sexual activity in warm waters. The density of individuals in tanks is the

greatest importance from the biotic factors [4,5]. The researcher and hirudotherapist Kamenev in his studies recommends replacing water once every 72 hours, thus having the lowest mortality of leeches [4]. The work of a few authors [3] established the optimal temperature conditions for pairing and laying cocoons: $+22-25^{\circ}\text{C}$ and $+24^{\circ}\text{C}$, respectively. At temperatures below $+15-16^{\circ}\text{C}$, copulations are not recorded. In the Mikhailov's research was found that the slightly acidic (pH 5.0–6.5) environment is optimal for the normal life of a medical leech [5,6]. The mass of leeches affects the quality of the offspring indicated in the literature [3,7–10]. It should be noted that mating occurs in water and can be transverse or one-sided [11–14] and that factors affecting on the sexual behavior and mortality of

Table 1. Total number of animals with leeches’ mortality

Group of animals	Total number of animals at intercourse	Number of animals with clitella	Mortality of animals with clitella (number of animals)
1 <i>H. verbena</i>	140	110	0
2 <i>H. verbena</i>	140	115	18
3 <i>H. medicinalis</i>	60	52	1
4 <i>H. medicinalis</i>	60	49	7
Total number of animals	400	326	26

leeches, not only in vivo, but also in artificial conditions, remain largely unexplored. For example, we did not find out what will happen to medicinal leeches, which are not immediately allowed to breed in the peat-soil or other conditions after the appearance of fertilization belts. Therefore, our goal was to explore the time and conditions that may affect themselves and their offspring.

Materials and Methods

Group of animals

For the study, four experimental animals groups were formed: 1 control – medicinal leeches (*Hirudo verbana*), which were allowed to breed immediately after the appearance of clitella; 2 experimental – medicinal leeches (*H. verbana*), which were kept in an aqueous medium after the appearance of clitella



Figure 1. Dead leeches with clitella. White arrows indicate clitella: A,B,C,D.

Table 2. Survival of animals after landing for laying cocoons

Group of animals	% of mortality of leeches with fertility belts in the aquatic environment before landing on reproduction		% of mortality of animals after cocoon deposition	
	Control group	Experimental group	Control group	Experimental group
<i>H. verbena</i>	0.000091±0.0000002	15.6 ±2.1*	2.0±0.4	95.1±0.7*
<i>H. medicinalis</i>	0.000085±0.00000015	14.3±1.5*	1.5±0.2	93.2±0.9*

* – $p < 0,05$ in comparison with the control group

for a month and then allowed to breed; 3 control – medicinal leeches (*Hirudo medicinalis*), which were allowed to breed immediately after the appearance of the clitella; 4 experimental – medicinal leeches (*H. medicinalis*), which were kept for a month in an aqueous medium after the appearance of clitella for a month then allowed to breed.

Experiment scheme

Initially, medicinal leeches were placed in 4-liter sterile bottles glass with 2 liters of dechlorinated sediment water (four animals in each container) for 2–3 weeks for copulation at an ambient temperature of +22–24°C. The animals of the control and experimental groups had the water changed daily. This procedure obviates the contamination factor of leeches' metabolic waste, which on day 4 can lead to their death. Four leeches were kept in the container, which prevented the negative impact of density, which can also affect mortality. The indicators of the experimental animals' group were compared with the indicators of the control group. The control group was leeches, which were immediately allowed to breed. The experimental group was observed daily for a month after the appearance of clitella. We considered the mortality of leeches with fertilization zones before planting during the month, as well their survival rate after planting. Control groups immediately for 2–3 days after the appearance of fertility zones were allowed to reproduce. Offspring were analyzed after hatching out of the cocoons.

Bioethics

Animal manipulation was carried out in accordance with the rules and regulations for the treatment of laboratory animals: principles of bioethics, legislation and requirements in accordance with the provisions of the „European

Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes”, the Law of Ukraine „On the Protection of Animals from Animals handling”. Such a responsible commission received from the bioethicists of Zaporizhzhya National University protocol №1 from March 28, 2018.

Statistical analysis

Statistical data processing was performed using the computer program SPSS v.21.0. (IBM SPSS Statistics., USA). The selected parameters indicated in the table below have the following notation: X – the average value of the sample, SE – standard error of the average value of the sample. The significance of differences between the mean values was evaluated by the Student's criterion after checking the normal distribution. Differences were considered significant at $p < 0.05$.

Results

The result of the experimental study of sexually mature individuals of two species of medicinal leeches after copulation and appearance of clitella on their bodies, after three to four weeks in the water, their mortality averaged *H. verbana* $15.6 \pm 2\%$ and *H. medicinalis* $14.3 \pm 1.5\%$ compared with the control group where their mortality was 0.000091 ± 0.0000002 and 0.000085 ± 0.00000015 , respectively $p < 0.05$ (Table 1,2). Dead medicinal leeches had huge clitella on their bodies, which literally protrude (Fig. 1).

After their death, there were no injuries or defects on their bodies. Partial cannibalism was also not detected. Analyzing the mortality rate of sexually mature leeches during breeding in peat-soil environment in the experimental group was *H. verbana* $95.1 \pm 0.7\%$ and *H. medicinalis* $93.2 \pm$

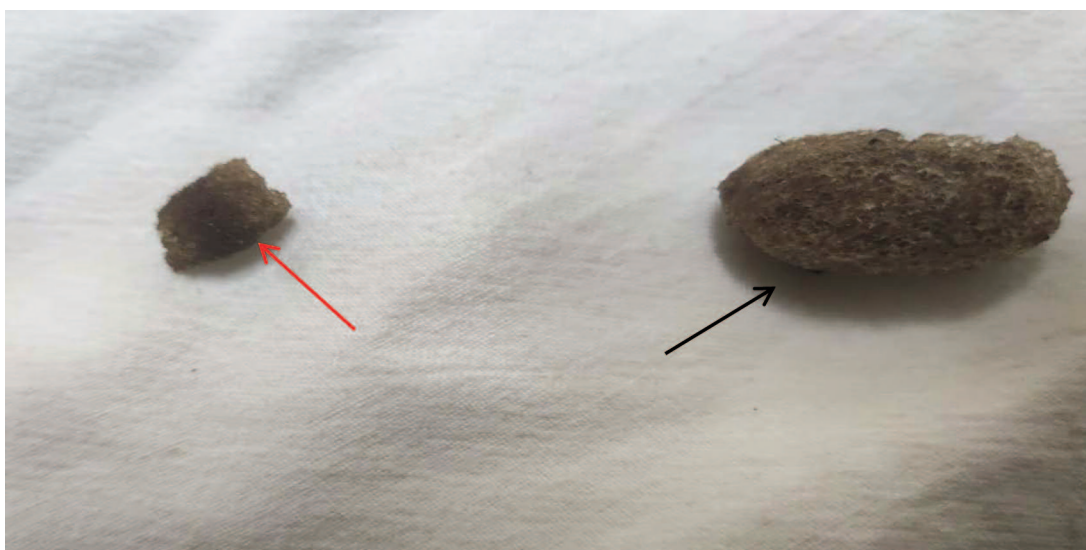


Figure 2. Cocoons from medical leeches *H. medicinalis*: red arrow – experimental group; black – control group

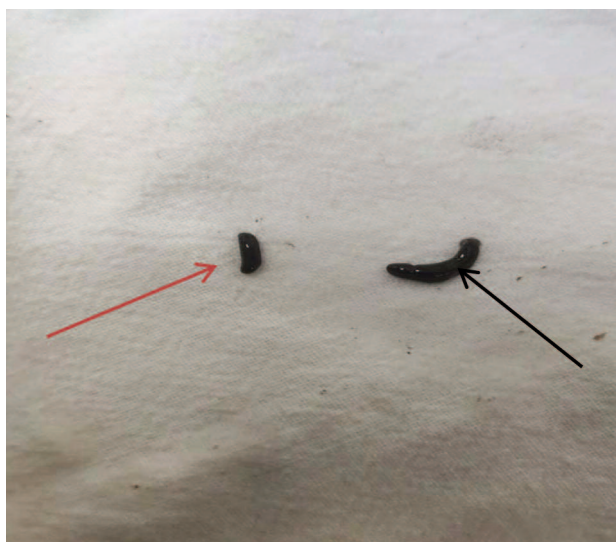


Figure 3. Sizes of offspring of medical leeches: the red arrow indicates the survived offspring of the experimental group; black – control group

0.9% compared with control groups $2.0 \pm 0.4\%$ and $1.5 \pm 0.2\%$, respectively $p < 0.05$ (Table 2).

Also, the experimental group of animals deposited defective cocoons compared with the control (Fig. 2). In addition, the experimental group of animals had a small number of live offspring, and the survivors were smaller than the ones from the control groups (Fig. 3).

In most cases the cocoons of the experimental animals' group were dead, and those that were alive died after one or two days (Fig. 4).

Discussion

Based on our results, the previous hypothesis is

put forward that the death of leeches with pronounced clitella may be due to necrotic intoxication. The result: inability to deposit the cocoon due to lack of air (peat-soil mixture). Fertile germ cells with nutrient material and accompanying structures for the cocoon accumulate in the genital tract of leeches. Undeveloped reproductive material, probably through the stages of apoptosis and subsequent necrosis, accumulates in the genital tract of the sexually mature individuals, causing acute inflammation and intoxication, with its subsequent death. We assumed that the oxygen-air medium and the surrounding substrate, which allows it to form a solid cocoon, can serve as a limiting factor. It should also be noted that clitella could disappear in some leeches. This may be because most leeches destroy these foreign particles, or the fertilization process is not complete. Other researchers describe that a certain percentage of leeches cannot cope with the consumed host blood and that they also die as a result [15–18]. But to confirm that mortality is associated not only with this factor but also with planting time and conditions, we have shown a comparison with a control group of animals, who had been placed in soil on time. In our previous studies, it was shown that leeches, with defective clitella, die when cocoons are deposited [19]. As a result of this, our studies can also be justified, but here also the defect of the body plays a role. According to our results, we can conclude that after the leeches are deposited for sexual intercourse, it is worth watching the appearance of clitella and after their appearance it is necessary to separate them from each other to lay cocoons as soon as possible,



Figure 4. Progeny of the experimental group A and B: red arrows indicate dead leeches of the experimental group

otherwise (if they are left in water), their death is possible. It should also be noted that in some leeches, slightly differentiated clitella disappeared, and leeches without them did not show mortality. In our previous studies and in the observations of other authors, leeches can manifest cannibalism [20,21], which may even lead to the death of a leeches, but in current study there were no signs of cannibalism which could be a death factor. Also, it should be noted that density and water factors did not affect animal mortality. First, the water was changed every day in the experimental and control groups, which makes its toxicity impossible. Secondly, there were four animals in the tank, which makes it impossible to influence the density factor. All indicators of the experimental group were compared with the control, which discards these two negative factors. Also, the peat-soil environment for placing leeches for laying cocoons was the same, which excludes the negative influence of the environment itself. All indicators of the experimental group were compared with the control. Further research will allow us to deepen understanding of the pathogenesis of individuals' death with pronounced clitella ready for laying cocoons.

In conclusions, our results show that under artificial conditions, if leeches are not released in time to lay cocoons (after the appearance of clitella), some leeches can die, and when bred, almost all.

The surviving part will give defective cocoons and mostly dead offspring. The obtained results can be important not only for biofactories engaged in breeding these animals, but also for zoologists, biologists, hirudotherapists, scientists working in this field, who want to preserve this species. And the obtained scientific results can reduce the process of extinction during breeding, which will increase their number. In the future it is planned to study clitella of dead leeches in details.

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