

Effects of various environments on number of cocoon and offspring in breeding of southern medicinal leech, *Hirudo verbana* Carena, 1820

Güney tıbbi sülügü, *Hirudo verbana* Carena, 1820 yetiştiriciliğinde farklı ortamların koza ve yavru sayısına etkisi

Bahadır Uğural^{1*} • Ramazan Serezli²

¹ İzmir Kâtip Çelebi University, Faculty of Fisheries, 35620, İzmir, Turkey

<https://orcid.org/0000-0001-7379-5826>

² İzmir Kâtip Çelebi University, Faculty of Fisheries, 35620, İzmir, Turkey

<https://orcid.org/0000-0003-4819-6607>

Corresponding author: bahadir.ugural@ikc.edu.tr

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Abstract: Breeding medicinal leeches in controlled environments is extremely important in order to prevent their extinction. Moreover, due to medicinal leeches coming into contact with the patient's blood, breeding medicinal leeches in hygienic conditions is essential to prevent possible complications.

In this study, breeding patterns of *Hirudo verbana* were studied in controlled conditions and the effects of various moist environments were examined. Peat, hydrogel and chopped sponge materials were compared in terms of number of cocoon and offspring in medicinal leech breeding. Peat and hydrogel environments didn't show any statistically significant difference for number of cocoon and offspring breeding. Cocoon per leech was 3.13 ± 0.74 for peat and 2.80 ± 0.56 for hydrogel respectively. Offspring per cocoon was 11.81 ± 2.27 for peat and 12.52 ± 1.98 for hydrogel ($P > 0.05$) respectively. In conclusion, hydrogel could be a new material for medicinal leech cocoon deposition environment especially for laboratory breeders.

Keywords: Medicinal leeches, cocoon, offspring, peat, hydrogel

Öz: Tıbbi sülüklerin kontrollü ortamda yetiştiriciliğinin yapılabilmesi, sülük neslinin tükenmesinin engellenmesi için büyük önem taşımaktadır. Ayrıca tıbbi sülüklerin, hijyenik koşullarda yetiştirilmesi, insan sağlığı için kullanım aşamasında istenmeyen komplikasyonların engellenmesi için gereklidir.

Bu çalışmada, *Hirudo verbana* türünün yetiştiriciliği kontrollü ortamlarda yürütülmüş ve çeşitli nemli ortamların etkileri incelenmiştir. Torf, hidrojel ve kırılmış süngerin koza ve koza içindeki yavru sayısı üzerine etkileri karşılaştırılmıştır. Torf ve hidrojel ortamları koza ve yavru sayısına etki edecek istatistiksel olarak anlamlı bir farklılık göstermemiştir. Sülük başına koza sayısı torf için 3.13 ± 0.74 , hidrojel için ise 2.80 ± 0.56 olarak belirlenmiştir. Koza başına yavru sayısı torf için 11.81 ± 2.27 hidrojel için ise 12.52 ± 1.98 olarak belirlenmiştir ($P > 0.05$). Sonuç olarak, hidrojel özellikle laboratuvar ortamında tıbbi sülük üretimi yapanlar için yeni bir koza bırakma ortamı materyali olabilecektir.

Anahtar kelimeler: Tıbbi sülük, koza, yavru, torf, hidrojel

INTRODUCTION

Medicinal leeches have been used for treatment for centuries and always have an economical value. *Hirudo verbana* is one of the most popular species after the *Hirudo medicinalis* used in medicine in Europe and Turkey (Elliott and Kutschera, 2011). Medicinal leech population of *Hirudo verbana* mostly exist in freshwater ecosystems in Turkey. Although Turkey's natural habitats and suitable climate provide optimum breeding conditions, excessive harvesting and the destruction of freshwater habitats lead to the extinction of medicinal leeches (Saglam, 2018). In order to prevent their worldwide extinction, medicinal leeches were put under protection in 183 countries by CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora). Also, only Turkey imposed specific export quota in order to prevent excessive harvesting (CITES, 2019).

The importance of medicinal leech breeding has increased with the decrease in population in natural resources. Leeches used for medical purposes come in direct contact with human blood in therapies, therefore hygienic breeding of this species is considered a priority for leech farmers (Gileva and Mumcuoglu, 2013). Under "Regulation on Traditional and Complementary Medicine Practices" published by the Turkish Ministry of Health on 27 October 2014, the need for medicinal leeches to be sterile and the importance of breeding them in such facilities were emphasized (Official gazette, 2014).

In medicinal leech natural living conditions, hibernation, cocoon laying and hatching of offspring leeches occur in peat or ground material in swampy areas (Kutschera and Elliott, 2014). In leech breeding facilities, organic-based materials such as peat and moss are extensively used for cocoon

deposition material. However, organic-based materials like peat are inherently rich in microorganisms (Küster, 1972). These environments may carry the risk of contaminating the laboratory and leeches. In this study, we examine the alternative cocoon deposition materials which are synthetic, reusable, hygienic, easy to clean and we compare the advantages and disadvantages of these materials with the most commonly used peat.

MATERIALS AND METHODS

Medicinal leeches

A total of 40 medicinal leeches (*Hirudo verbana* Carena, 1820) were bought from an Izmir based company which stated that the leeches collected in August 2016 from Yay lake, Develi district of Kayseri, Turkey. Leeches heavier than 5 g were selected. Smallest leech was 5 g while the largest was 13.2 g, averaging 7.62 ± 2.13 ($X \pm SD$) g. Also, leeches examined morphologically, any wounded or swelled (in case of recently fed) leeches were not used in the experiment.

Cocoon deposition environment

In leech breeding experiments mostly moist peat (Petrauskiene et al., 2009; Ceylan et al., 2015; Saglam, 2017) and moist moss (Malek et al., 2019) were used for leeches to lay cocoons. In this experiment two other materials offered for cocoon deposition environment; chopped sponge and hydrogel.

The chopped sponge is generally used as filler in the furniture industry. Before use, the chopped sponges were well-washed and dried. After placing in jars, water added slowly on top to get moisty environment.

The hydrogel is a super absorbent material made from cross-linked polyacrylamides and cross-linked acrylamide-acrylate copolymer (Narjary et al., 2013; Ahmed, 2015). The hydrogel can absorb water up to 500-600 times of their weight and release water if the environment dries up (Abobatta, 2018).

Experiment

The study conducted in aquatic sciences laboratory in Izmir Katip Çelebi University, Faculty of Fisheries between 22 August 2016 and 12 January 2017. The experiment was designed based on previous studies (Wilkin and Scofield, 1991; Spencer and Jones, 2007; Petrauskiene et al., 2009; Ceylan et al., 2015). The trial consists of three stages. At the first stage leeches kept in a half-filled aquarium (100×50×40 cm) in 100 L water for 15 days to ensure they adapt to new conditions. The same aquarium used after feeding for digestion for 32 days. The water temperature was 20 ± 1 °C during adaption and digestion periods. Leeches were fed with warm bovine blood (37 ± 1 °C) via blood sausages (McLoughlin and Davies, 1996; Kutschera and Elliott, 2014). Blood clot and plasma were mixed with a blender for homogenisation before filling blood sausages, no additional anticoagulant used. Chlorine free natural tap water used during experiment, water

parameters were pH 8-8.2, NH₃-N (mg/L)<0.001, NH₃ (mg/L)<0.01, dissolved O₂ 7.31 mg/L.

The second stage was copulation, leeches transferred to five 5 L plastic jars. Every jar filled with 3 L water, 8 leeches kept in per jar. Every 3 days water replaced with water of same temperature. During water replacement, leeches collected in one place and randomly selected 8 leeches were once more placed in the jars. Copulation lasts after 3 weeks at 25 ± 0.5 °C. Then, discolouration and swelling appeared on leeches'abdomen around clitellum indicating that leeches ready to deposit cocoons (Wilkin, 1989; Elliott, 2008; Zhang et al., 2008).

After copulation, the third stage was cocoon deposition. Leeches were transferred to eight 5 L jars which were filled with moist materials. 5 leeches put in per jar. 2 of the jars filled with chopped sponge, 3 of them filled with moist peat and other 3 filled with large-grained hydrogel. Leeches were distributed to groups in equal weight and size. The mean weight of the leeches in groups was 7.56 ± 2.31 g in peat group, 7.52 ± 2.22 g in the hydrogel group, and 7.88 ± 1.90 g in sponge group respectively. There was no statistically significant difference between groups for broodstock leech weight ($P>0.05$). All jars were covered with a dark plastic cover. Cocoon deposition occurred 45 days at 25 ± 0.5 °C. Cocoons were checked and collected 2 times per week. Cocoons collected into different jars which have hydrogel in them and labeled according to the material in the same conditions. The trial lasted until all cocoons hatched in 30 days.

Statistical Analysis

The data were analyzed with SPSS version 24.0 for Windows (IBM Corp., Armonk, NY, USA). Oneway ANOVA used for weight analyzes, Chi-square test was used for categorical distributions, T-Test was used for normal distributions and Mann-Whitney U Test used for non-normal distributions.

RESULTS

When leeches were transferred to moist materials, they were quickly burrowing into peat and hydrogel, but leeches placed on the sponge remained on the sponge pieces for a long time, and then they slowly moved to the bottom of the jar between the sponges, which may indicate that the sponge does not provide a favorable habitat for leeches.

In the experiment with moist sponge filled jars, it was observed that there was a bad smell at the end of the first week. On day 10, a total of 4 leeches were found dead. NH₃-N and NH₃ analysis of the water at the bottom of the sponge environment were higher than 8.5 mg/L. Because of the unfavorable environment, irritated leeches and the dead leeches, experiment called off for sponge environment on day 10. A total of 15 cocoons were taken from the sponge environment (Figure 1), 5 of these cocoons was unfertilized.

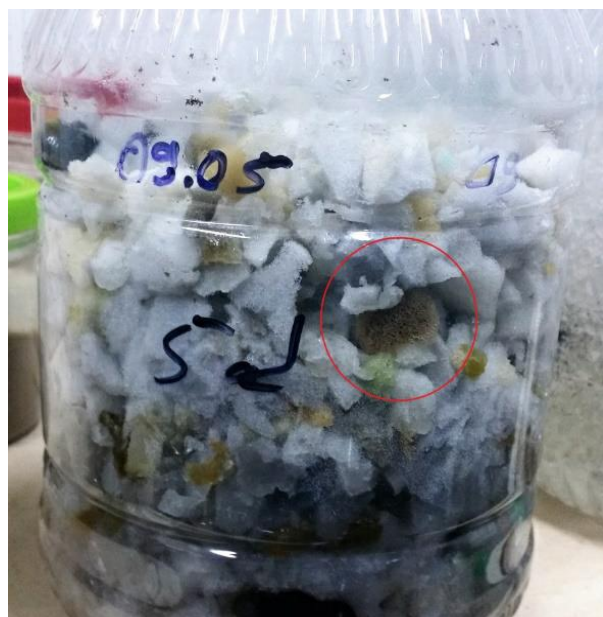


Figure 1. Sponge environment. The red circle is showing a cocoon

In experiment with moist peat, peat was poured out from jars to find cocoons inside once a week. A total of 47 cocoons with the average per leech 3.13 ± 0.74 were taken from the moist peat environment (Figure 2).



Figure 2. Moist peat environment. The bold circle is showing a new deposited cocoon with white foam. The other circles are showing the cocoons in the peat

In experiment of hydrogel environment, it was observed that leeches deposited the cocoons to the top of the hydrogel. Eliminating the need for pouring out the material made it easy to collect cocoons, saved time and provided a clean environment. A total of 42 cocoons with the average per leech 2.80 ± 0.56 were taken from the hydrogel environment (Figure 3).

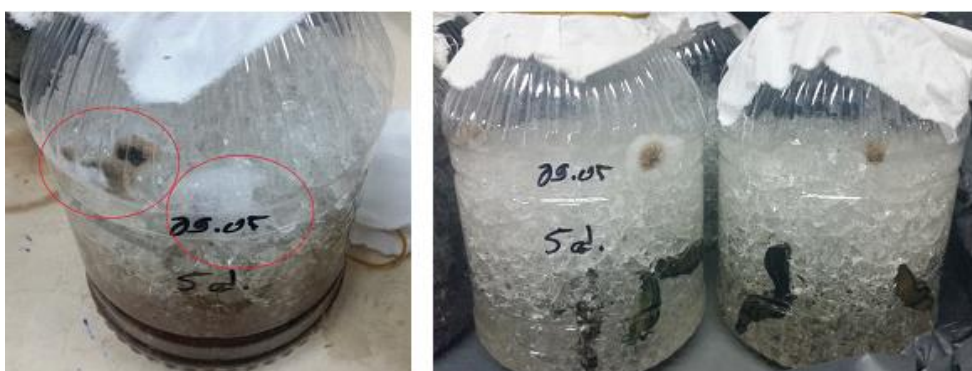


Figure 3. Hydrogel environment. The circle on the right is showing a new deposited cocoon with white foam, the other circle is showing the older cocoons

After the sponge environment failed, only the data related the environments of peat and hydrogel were used for statistical analysis.

It was determined that there were 8.04 ± 5.87 (0 to 16) offspring per cocoon in the peat group and 7.45 ± 6.40 (0 to 17) offspring per cocoon in the hydrogel group. The difference between groups was not statistically significant for offspring number per cocoon ($P > 0.05$). When cocoons with and without offspring were compared between the groups, 15 of 47

cocoons were empty in the peat group and 17 of 42 cocoons in the hydrogel group were empty.

There was no statistically significant difference between groups for rate of the empty cocoons ($\chi^2 = 0.716$ $P > 0.05$).

When the cocoons with offspring were compared between the groups, average offspring per cocoon was 11.81 ± 2.27 (7 to 16) in the peat group and 12.52 ± 1.98 (8 to 17) in the hydrogel group. Statistically there was no significant difference found in groups for offspring number per cocoon ($P > 0.05$).

Table 1. The broodstock weight, mortality, hatching rate, cocoon and offspring efficiency according to the groups

Groups ¹	Weight ² (g)	Mortality ³ (%)	# C per B ⁴ (n)	# O per C ⁵ (n)	Hatching ⁶ (%)	#O per CWO ⁷ (n)
Peat	7.56 ± 2.31	0	3.13 ± 0.74	8.04 ± 5.87	68.09	11.81 ± 2.27
Hydrogel	7.52 ± 2.22	0	2.80 ± 0.56	7.45 ± 6.40	59.52	12.52 ± 1.98
C. sponge	7.88 ± 1.90	40	1.5 ± 0.52	6.53 ± 4.88	33.33	9.80 ± 1.23

¹ Cocoon deposition environment, ² Weight of the broodstock, ³ Dead broodstock rate, ⁴ Cocoon number per broodstock, ⁵ Offspring number per cocoon, ⁶ Hatching rate of cocoon, ⁷ Number of offspring per cocoon for cocoons with offspring

DISCUSSION

Medicinal leech breeding has always been a popular topic due to its human medicinal use. Feeding, growth, reproduction and survival of medicinal leeches have been studied under various physical environmental parameters. In previous studies, peat is the most used cocoon deposition material for leeches. In this study, we studied different synthetic materials beside peat for cocoon deposition environment. We found similar results with [Ceylan et al. \(2015\)](#) and [Petrauskiene et al. \(2009\)](#) for cocoon and offspring numbers of Southern Medicinal Leech (*Hirudo verbana* Carena, 1820). [Ceylan et al. \(2015\)](#) observed in their study, leeches deposit the average 3.20 ± 1.87 cocoons and the average offspring from each cocoon was 12.29 ± 5.14 . [Petrauskiene et al. \(2009\)](#) reported the average of 3.29 ± 0.277 cocoons per leech and the average 10.45 ± 0.710 offspring per cocoon were obtained. In our study, the average cocoons taken from per leech was 3.13 ± 0.74 and the average offspring from each cocoon was 11.81 ± 2.27 in peat environment. In hydrogel environment, the average cocoons taken from per leech was 2.80 ± 0.56 and the average offspring from each cocoon was 12.52 ± 1.98 . In the sponge environment, the experiment was terminated on the 10th day due to deaths and degradation of the environment, but 15 cocoons and 98 newborns were bred within 10 days. [Manav et al. \(2019\)](#) emphasized 10% of 40 cocoons were empty (without offspring) which were obtained from the broodstock fed with cattle blood and 26% of 100 cocoons were empty which were obtained from broodstock fed with chicken blood. In our study, leeches only fed with bovine blood but 31.91% of 47 cocoons were empty in peat environment and 40.47% of 42 cocoons were empty in hydrogel environment. In the study of [Manav et al. \(2019\)](#) southern medicinal leeches supplied from a laboratory while offspring and cultivated in laboratory and fed with specific blood samples every month during 8 months. In our study leeches fed only one time after captured from nature. The difference on reproductive efficiency of two studies should derive from feeding regime of leeches because it isn't possible to know earlier feeding frequency or feed quality of wild leeches. The feeding regime (frequency, origin and quantity of blood meal) may affected the reproductive efficiency of medicinal leeches.

Disadvantages of using traditionally used peat in laboratory conditions; it is difficult to perform visual control of cocoons and offspring release during reproduction, checking cocoons creates dirt in every control and peat needs to be changed for every new breeding. In addition, there is a possibility that the peat may deteriorate and cause contamination due to its organic structure. Leeches may die in peat and it is not possible to determine dead leeches in peat until pouring the peat for control ([Petrauskiene et al., 2009](#)). Death may cause deterioration of peat.

As an advantage, peat has the highest numbers of cocoons and offspring for leech reproduction even though the difference was not statistically significant ($P > 0.05$). Natural behaviour of medicinal leeches to deposit their cocoons dark, damp places into moist soil on terrestrial habitat ([Kutschera and Roth, 2006](#); [Kutschera and Shain, 2019](#)). Cocoons initially surrounded by a foamy substance then take the final solid form ([Saidel et al., 2018](#)) like an egg covered by thin sponge. [Saidel et al. \(2018\)](#) thought this three-dimensional structure of cocoon protects itself from desiccation and displacement. Also leeches may have an instinct to protect their cocoons by depositing them into moist soil. Leeches must find or make a gap for placing cocoon ([Figure 4](#)).



Figure 4. Recently deposited cocoon in peat material. Leech must have made a gap before depositing cocoon. The gap could be seen around the white foam

This could be the answer why peat environment has the higher number of cocoon than hydrogel and why leeches placed cocoons on top of the hydrogel. Broodstock leeches in hydrogel may lose time to search for a gap and it is not possible to make a gap in hydrogel because of its slippery surface. Besides working disadvantages of peat, it may be the best cocoon deposition material for leech's instinct as a natural cocoon deposition area in nature for medicinal leech populations. The hydrogel environment provides a clean environment while controlling cocoon deposition and offspring getting out of the cocoon. Leeches lay their cocoons on top of the hydrogel environment. Dark cover around the jars may have a role by giving the feel of a gap to leeches so they deposit cocoons on top of hydrogel material. The hydrogel is a transparent material so it is easy to detect cocoons and dead leeches. The hydrogel

is washable and can be used for long periods of time which also provides another advantage for laboratory conditions. In conclusion, although the cocoon efficiency is higher in the peat material, hydrogel could be used as an alternative material for leech cocoon deposition environment in leech breeding sector.

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