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RESEARCH ARTICLE

Impact of selected hormonal agents on the effectiveness of controlled reproduction of cultivated female European grayling

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Abstract. The suitability of the hormonal agents Ovopel (one granule kg^{-1} female body weight), human chorionic gonadotropin – hCG (1000 IU kg^{-1} female body weight), and carp pituitary homogenate – CPH (3 mg kg^{-1} female body weight) for use in the controlled reproduction of female European grayling, *Thymallus thymallus* (L.), was tested. The fish from the control group were administered injections of saline solution (0.5 cm^3 kg^{-1} female body weight). The agents were administered in single intraperitoneal injections. The effectiveness of Ovopel and CPH injections was 100%, while hCG stimulation resulted in ovulation in 90% of the females. Just 40% of the females ovulated in the control group. The total latency time from injection to ovulation in the last females which ovulated was 87.9°D. Embryo survival to the eyed egg stage was 29.23% for Ovopel, 20.22% for hCG, 14.80% for CPH, and 47.50% in the control group. The highest spawning effectiveness coefficient (Se) was confirmed in the group of fish administered Ovopel (0.29), while the lowest was was in the CPH group (0.15). Following the administration of hCG, the Se was 0.18, while in the control group it was 0.19. Hormonal stimulation was not noted to have had a negative impact on spawner survival.

Keywords: European grayling, cultured brood stock, hormonal stimulation, hCG, CPH, Ovopel

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Introduction

European grayling, *Thymallus thymallus* (L.), is a typical rheophilic species (Mallet et al. 2000). Because of its specific environmental requirements, it is susceptible to negative changes in the environment it inhabits, and this leads to the limited abundance of many natural populations of this species. One of the ways to counteract this trend is to stock open waters with material obtained under controlled conditions. Salmonid hatcheries should play a leading role in the production such material (Witkowski and Bartel 1999). The biotechnology for propagating this species is not yet fully developed, and maintaining maternal grayling broodstocks under controlled conditions still faces many challenges (Kowalewski 1987, 2002, Goryczko et al. 1995). One of the most important of which is high spawner mortality rates, especially in the periods prior to, during, and following spawning. This is primarily because of the necessity of monitoring spawner maturity, which requires

handling the fish repeatedly during the spawning period. The spawners are particularly sensitive to all types of skin injuries that can lead to secondary bacterial and fungal infections resulting in losses of as much as 60% of broodstocks (Grudniewska and Szmyt 2003).

One possible solution is to use hormonal agents to stimulate reproduction. This practice is well established with commercially valuable cyprinid species (Kozłowski 1994, Drori et al. 1994, Brzuska 2000), as well as with other fish species: European seabass, *Dicentrarchus labrax* (L.); (Mylonas et al. 2003); turbot, *Scophthalmus maximus* (L.), (Mugnier et al. 2000); perch, *Perca fluviatilis* L., (Kucharczyk et al. 1996, 1998); pike, *Esox lucius* L., (Szabó 2003); pikeperch, *Sander lucioperca* (L.), (Zakęś and Demska-Zakęś 2005). While it is not difficult to obtain eggs from salmonid females thanks to their anatomical structure, extended spawning periods can pose problems. The application of hormonal stimulation not only induces ovulation, but more importantly it can be used to synchronize spawning (Arabaci et al. 2004). Achieving similar effects in the reproduction of grayling spawners is the next step in developing the necessary biotechnology for propagating this species under controlled conditions.

The primary focus of this study was to determine the possibilities of using selected hormonal agents during the controlled spawning of European grayling with a particular focus on the effectiveness of their application, the reaction time of the grayling to the selected agents, and the survival of the embryos to the eyed egg stage.

Materials and methods

The study material comprised grayling spawners from a maternal broodstock which has been maintained at the Department of Salmonid Research in Rutki, (IFI Olsztyn) since 1990 (Grudniewska and Grudniewski 1991, Goryczko et al. 1995). Because of the significant aggressive behavior exhibited by grayling males following Ovopel injections observed in previous studies (Szmyt et al. 2007), only females aged 2+ which were spawning for the first time were subjected to hormonal stimulation.

The suitability for use in controlled grayling reproduction of the hormonal agent Ovopel (Unic-trade, Hungary) (group I), human chorionic gonadotropin – hCG (Biogonadyl, Biomed Lublin, Poland) (group II), and carp pituitary homogenate – CPH (Argent, USA) (group III) was tested. One granule of Ovopel contains 18-20 μg GnRH analog (D-Ala^6, Pro^{9}Net-mGnRH) and 8-10 mg of metoclopramide, a dopamine inhibitor (Horvath et al. 1997). The agents were applied in single intraperitoneal injections in the following doses: Ovopel – one granule kg^{-1} female body weight, hCG – 1000 IU kg^{-1} female body weight, and CPH – 3 mg dry weight kg^{-1} female body weight. Before administering the intraperitoneal injection, the tested hormonal agent was dissolved or pulverized in a sterile 0.9% NaCL solution (Thalathiah et al. 1988, Kucharczyk et al. 1996, Horvath et al. 1997) and then prepared for application in dilutions of 0.5 cm^3 kg^{-1} female body weight. Thirteen females were selected at random to receive stimulation with Ovopel and ten females each were selected at random to receive stimulation with human chorionic gonadotropin and carp pituitary homogenate. The control group (10 individuals) comprised females injected once with saline solution in the amount of 0.5 cm^3 kg^{-1} female body weight (group IV) (Table 1). In order to determine precisely the optimal dose of the ovulation stimulation agent, the weight of each female was determined to the nearest 0.1 g.

The water temperature on the day of the injection was 6.2°C, and this increased systematically throughout the experiment (Fig. 1). The effectiveness of the stimulation procedure was monitored four times on days 3, 6, 10, and 13 following injection. Each time the spawners were examined, approximately 100 eggs were collected from the ovulating females. The sex products from individual females were collected once only without repeated stripping. After spawning, all of the fish were placed in one tank without being divided into experimental groups. Each sample of eggs collected was fertilized separately in Petri dishes with semen collected from three males in excess in relation to the quantity of eggs. The after the eggs had swelled

Table 1
Values (mean ± SD) and range of female European grayling, *Thymallus thymallus*, body weights in different variants of the experiment. The mean body weights of the fish did not differ significantly statistically among the experimental groups (ANOVA, $P > 0.05$)

		Body weight (g)	
Hormonal agent	Number of fish	mean (± SD)	Range
Ovopel	13	160.9 (± 40.4)	69.5 - 209.9
hCG	10	181.7 (± 74.5)	60.4 - 320.2
CPH	10	204.6 (± 42.1)	161.0 - 310.0
Saline solution	10	146.2 (± 32.2)	93.7 - 191.4

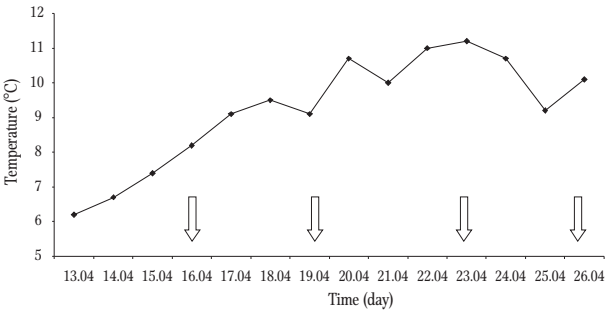

Figure 1. Water temperature during the experiment. Arrows indicate the dates when female European grayling, *Thymallus thymallus*, were examined.

from hydration, they were removed to troughs in California-type hatching trays.

During incubation, which was performed in groundwater at a constant temperature of 8°C, prophylactic baths with a formalin solution (concentration of 1:500 for 30 min), were applied at two-day intervals to prevent mold from growing on the eggs (Witkowski et al. 1984). At the end of the experiment the eyed eggs were separated from the dead eggs and survival was calculated.

The spawning effectiveness coefficient Se was also calculated (Kucharczyk et al. 1996):

$$S_e = O_s \times J_s$$

where:

O_s – ovulation success (quantity of ovulating females/ quantity of all females in the experimental group)

J_s – incubation success (quantity of live embryos in the eyed egg stage/ total quantity of incubated eggs).

The latency period of grayling females administered the different agents was determined as the percentage share of ovulating fish during subsequent spawner examinations in comparison to all of the fish in a given group. The quality of the eggs obtained was defined as embryo survival to the eyed egg stage. To obtain a full picture of the impact hormonal stimulation had on the quality of grayling eggs, it was determined whether or not there was a link between the latency period from injection to ovulation and the survival of embryos to the eyed egg stage.

Stress was minimized and the possibility of injuring the spawners was limited during all manipulations of the grayling for hormonal stimulation and stripping by first anesthetizing the fish in an aqueous solution of Propiscin at a concentration of 0.5 cm^3 per liter of water (Kazuń and Siwicki 2001).

The results were analyzed statistically with Statistica 9.0 (Stat Soft, Inc., USA). Single factor analysis of variance (ANOVA) was used to analyze the suitability of the different hormonal agents in grayling controlled reproduction. When the results of these analyses permitted rejecting H_0, Duncan's post-hoc test was used. Two-way analysis of variance was used to determine whether there were dependencies between the latency period between the administration of the hormonal agent and ovulation and egg survival. Differences were considered statistically significant at $P < 0.05$.

Results

During the experiment, no adverse effects were noted from the injections on spawner survival. Additionally, no fish deaths were noted between the moment of injection and ovulation. Ovopel and carp pituitary homogenate (CPH) induced ovulation in 100% of the fish treated. Human chorionic gonadotropin (hCG) induced ovulation in 90% of the female grayling treated, while ovulation occurred in 40% of the females that received injections of saline solution (control group). The latency period between injection and the beginning of ovulation in the last females to do so was 87.9°D (Fig. 2). When the spawners were examined again a fourth time after a subsequent 30°D, the share of ovulating females among those which had not yet done so had not increased.

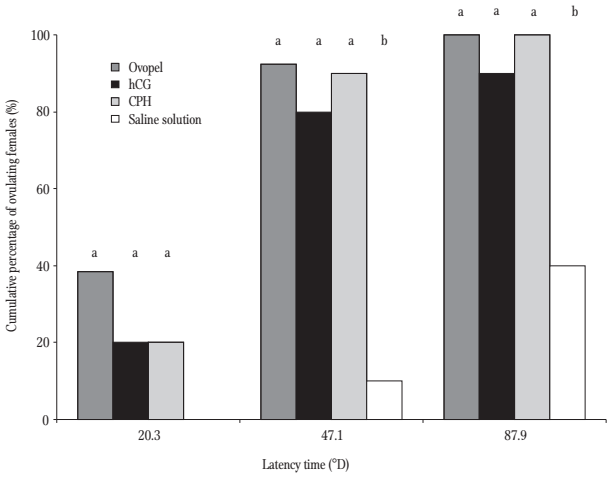

Figure 2. Impact of hormonal stimulation on the latency time of female European grayling, *Thymallus thymallus*, ovulation. Data presented are cumulative. No statistically significant differences were noted among the groups tagged with the same letter index (P > 0.05).

During the first examination at 20.3°D after hormonal injection, the most ovulating females were noted in group I (Ovopel) at 38%; however, there were no ovulating females in group IV (control). In groups II (hCG) and III (CPH) 20% of the females were ovulating (Fig. 2). No statistically significant differences (P > 0.05) were noted among the number of ovulating females treated with the different hormonal agents. The highest shares of ovulating females were noted in all treatment groups (except the control group) at the second spawner examination after a subsequent 26.8°D at values of 54% in group I (Ovopel), 60% in group II (hCG), and 70% in group III (CPH). The share of ovulating females in the control group was 10% (Fig. 2). Statistically significant differences (P < 0.05) were noted between the groups of fish treated with hormonal agents (Ovopel, hCG, CPH) and the control group. No statistically significant differences were noted in the number of ovulating females among the three experimental groups (P > 0.05). During the third examination of the spawners after a subsequent 40.8°D, in group I (Ovopel) 8% of the females were ovulating, while in groups II (hCG) and III (CPH) this figure was 10%. In the control group 30% of the females were ovulating (Fig. 2). These differences were not statistically significant (P > 0.05).

The highest embryo survival to the eyed egg stage was noted in the control group and was 47.5%, while it was the lowest in group III (CPH) at 14.8% (Fig. 3). Analysis of variance did not indicate any statistically significant differences (P > 0.05) in embryo survival depending on the hormonal agent used. In experimental groups I and II (Ovopel and hCG), the highest survival was in eggs obtained during the first examination of the spawners and 20.3°D after the hormonal injections (Figs. 4 and 5). Mean survival was 53.0 and 45.5%, respectively, in groups I and II. The survival of the eggs obtained during subsequent examinations at 47.1 and 87.9°D was decidedly lower at a mean of 12.4% (Fig. 4) and 28.0% (Fig. 5) in group I (Ovopel), respectively, while in group II

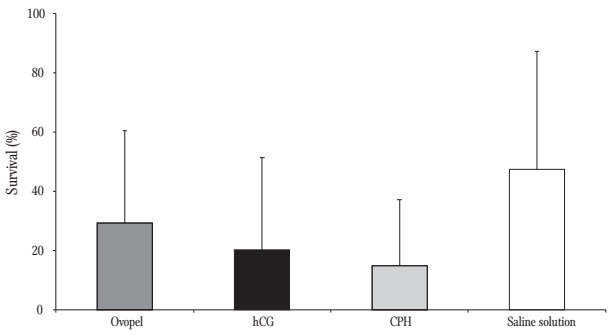

Figure 3. European grayling, *Thymallus thymallus*, embryo survival (mean ± SD) to the eyed egg stage depending on the hormonal agent administered.

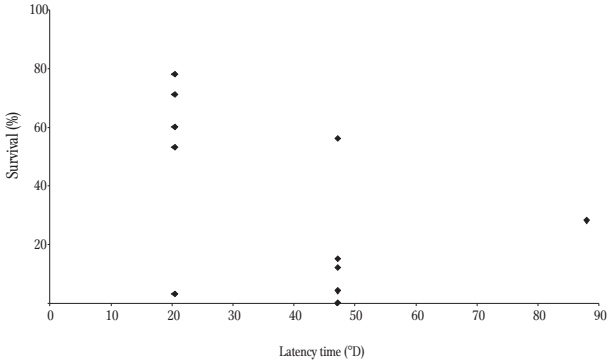

Figure 4. Impact of latency time from the injection of Ovopel to female European grayling, *Thymallus thymallus*, ovulation on the survival of embryos to the eyed egg stage in subsequent stages of the experiment.

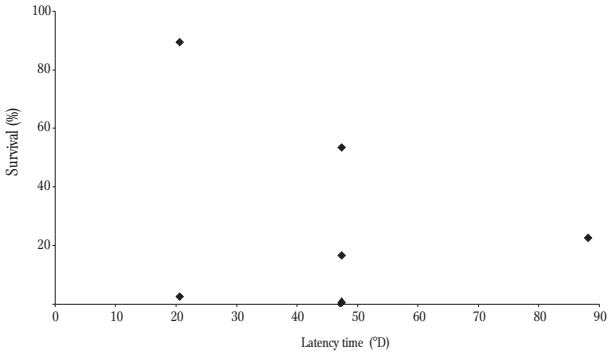

Figure 5. Impact of latency time from the injection of hCG to female European grayling, *Thymallus thymallus*, ovulation on the survival of embryos to the eyed egg stage in subsequent stages of the experiment.

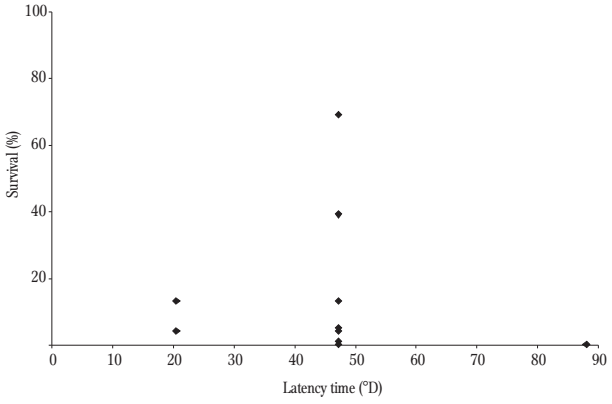

Figure 6. Impact of latency time from the injection of CPH to female European grayling, *Thymallus thymallus*, ovulation on the survival of embryos to the eyed egg stage in subsequent stages of the experiment.

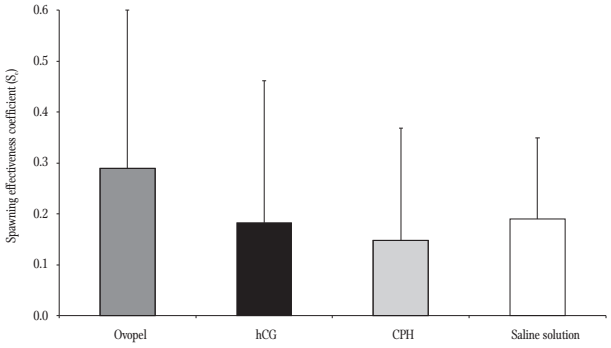

Figure 7. Spawning effectiveness coefficient S_e (± SD) in different experimental groups.

(hCG) it was 11.5 and 22.0%, respectively. In group III (CPH), the highest mean survival of 18.7% was confirmed the second examination of female maturity 47.1°D following injection with the hormonal agent, while the lowest survival was noted in this group 87.9°D following injection when no embryos survived (Fig. 6). The eggs obtained during the first examination of spawner maturity 20.3°D after the hormonal treatment was of median survival at 8.5%. Embryo survival to the eyed egg stage was the highest at 78.0% in the control group 47.1°D following injections, but it was only at a mean of 38.0% 87.9°D following the injections. No statistically significant dependencies ($P > 0.05$) were noted with egg survival and stripping time and the hormonal agent used.

The highest spawning effectiveness coefficient (S_e) was noted in the group of fish stimulated with Ovopel ($S_e = 0.29$), while the lowest was noted in the group of fish stimulated with CPH ($S_e = 0.15$). The values of the spawning effectiveness coefficient obtained in the other two groups were intermediate and close to each other (Fig. 7).

Discussion

The European grayling has not been a frequent subject in research on the hormonal induction of ovulation or spermiation. Carp pituitary and synthetic GnRH analogue have been used in these studies (Mikołajczyk et al. 2008). The authors, who used

synthetic GnRH analogue (Gonazon^{TM}) in doses of 16 and 32 μg kg^{-1} female body weight, obtained cumulative percentages of ovulating females of 81.4 and 74.0%, respectively. These results are comparable to those obtained in the current study, despite the fact that other hormonal agents were used.

The effectiveness of the hormonal stimulants applied depends on temperature. According to Pokorný and Kouřil (1999), one can anticipate induced ovulation of approximately 80% from females that have been injected with GnRH analog and carp pituitary extract and held at water temperatures of 7-8°C after about 29-50°D. Thus, ideally spawners should be held in systems with controlled environmental parameters following injection with hormonal stimulants (Kujawa et al. 1999). Such a study was conducted by Pokropski (2005), who held fish in waters at a constant temperature of 10°C following the injection of stimulants. The author stimulated wild grayling spawners with Ovopel, hCG, and carp pituitary, following which 80, 70, and 60% of the females ovulated, respectively, in the different experimental groups. The author confirmed ovulation in just 20% of the females in the control group.

Full control over environmental parameters is not always possible under production conditions in salmonid hatcheries, and the distribution of mean daily temperatures is reflected in the quantities of ovulating females. In the current study, an increased water temperature to 9°C resulted in the highest number of ovulating females in all of the experimental groups. Similar results, but at a temperature of 10°C, are reported by Randák et al. (2000). Randák (2002) also confirmed a positive correlation between increased water temperature and the efficacy of carp pituitary for inducing ovulation in females, although no such observations were noted with regard to Kobarelin, which is another GnRH analog. The author reported 100% effectiveness with regard to the first hormonal agent, and 95.5% for the second. Surprisingly high percentages of ovulating females were also reported for the two control groups with 82.4% for the groups that received no injection and 85.0% for the group that was injected with saline solution. These data were not reflected by the results of the current study or in the experiment conducted by Kouřil et al. (1987a, 1987b), in which the share of ovulating females ranged from 17 to 40%.

Kouřil et al. (1987b) analyzed the effectiveness of a synthetic analog of salmon GnRH and carp pituitary depending on temperature, dosage, and number of doses and a combination of these agents. According to these authors, the best results were achieved when both agents were used simultaneously in dosages of 25 μg analog GnRH kg^{-1} female body weight and 5 mg pituitary kg^{-1} female body weight, at a temperature range of 4-8°C. The shortest latency period between injection and ovulation and fully effective treatment was obtained under these conditions. Similar studies were conducted by Kouřil et al. (1987a), in which they tested carp pituitary and a synthetic LH-RH analog together. Fully effective stimulation and the shortest latency period from injection to ovulation of 34.4°D was again obtained only in the 4-8°C temperature range. The best effectiveness was obtained using carp pituitary in two doses of 1 and 4 mg kg^{-1} female body weight administered at a one-day interval. The results obtained in the present study regarding the number of °D from the administration of the hormone agent to ovulation are higher than those from the cited authors. This is likely to have been the result of the different hormonal agents used as well different daily dynamics in temperature changes.

Jungwirth (1979) also applied carp pituitary to stimulate female huchen, *Hucho hucho* (L.). This author compared the effectiveness of the agent dependent on the frequency of its administration. The greater effectiveness of two separate doses as opposed to a single dose (summarily equivalent) was especially evident in improved ovulation synchronization, but it was also noted that the females were easier to strip, the percentage of fertilized eggs was higher, and embryo mortality was lower.

There is little data in the literature regarding the impact of hormonal stimulation on the survival of grayling eggs during incubation. Pokropski (2005), who studied the impact of hormonal stimulation on the effectiveness of grayling reproduction, obtained different, although decidedly higher, embryo survival

rates to the eyed egg stage than those in the present study. The author reported survival rates of 69.3% in the group stimulated with hCG, 60.0% in the group stimulated with Ovopel, and 49.6% in the group stimulated with carp pituitary, while survival in the control group was 58.3%. These relatively high embryo survival rates are probably because the spawn was obtained from spawners caught in the natural environment and also because the fish were held at a constant and relatively high temperature of 10°C.

Grayling embryo survival was also studied by Randák et al. (2000) and Randák (2002). However, these authors only reported overall losses of incubated eggs from all of the females combined, but without specific information on the mortality linked to each hormonal agent. According to these authors, egg mortality was from 15 to 50%. Despite everything, these are much lower than those noted in the current experiment. One explanation for this might be that in the current study fish from younger age groups were used, and they were fed trout granulate exclusively. The eggs from such fish are generally of lesser quality than those obtained from older spawners, the diet of which is supplemented with larval flies, for example (Szmyt et al. 2004). The highest egg mortality was obtained in the control group, but it must be emphasized that only four of the ten spawners in this group spawned, and this could have impacted the reliability and legibility of these results. Lastly, the survival of the eggs of one female was exceptionally high, which could have had an impact on the mean results from this group.

It was attempted to link embryo survival to the eyed egg stage with the latency period from hormonal agent injection to ovulation. Despite such a dependence not having been noted, the results of the group stimulated with Ovopel are noteworthy; that this hormonal agent had a positive impact on egg survival is confirmed by the fact that the eggs obtained from the fish stimulated with this agent had the lowest mortality rates. Considering the spawning effectiveness coefficient (S_e), the results of the present study correspond best with the data published by Kucharczyk et al. (1997c). These authors used various hormonal agents to stimulate rudd, *Scardinius erythrophthalmus* (L.), and obtained results of the S_e coefficient in the range of 0.15-0.26. Similar results following stimulation were obtained during the out-of-season reproduction of common bream, *Abramis brama* (L.), injected with a series of human chorionic gonadotropin and carp pituitary (Kucharczyk et al. 1997b). The value of this coefficient was 0.19 for the higher and 0.11 for the lower doses of hormone. Szczerbowski et al. (2000) obtained similar results (S_e = 0.2) with perch stimulated with Ovopel. However, in this case, it was the lowest confirmed value for all of the stimulation agents. Decidedly higher values for this coefficient were obtained for perch (Kucharczyk et al. 1996) and for common bream (Kucharczyk et al. 1997a) that reproduced during the spawning season.

Applying selected hormonal agents to induce ovulation in controlled grayling reproduction can produce the desired results. Choosing the proper agent ensures obtaining good quality eggs and also shortens the time in which they can be obtained. This reduces the manipulation the spawners are subjected to thus limiting their mortality. Among the agents tested, Ovopel has the greatest potential for application in fish propagation. The results obtained indicate that this agent is relatively easy to administer, it is highly effective, and there are no negative aspects to its application.

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Author contributions. M.S., S.D., J.G., and D.K. designed and performed the experiment. M.S., S.D., and A.M.L. analyzed the data and wrote the paper.

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