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Steroid Hormone Pollution and Life History Strategies of Freshwater Planarians and Snails in a Mesocosm Experiment

Marcin Weselak and Anita Kaliszewicz *

Institute of Biological Sciences, Cardinal Stefan Wyszynski University in Warsaw, Wóycickiego 1/3, 01-938 Warsaw, Poland; marcinwese@wp.pl
* Correspondence: a.kaliszewicz@uksw.edu.pl

Abstract

The problem of steroid hormones in the aquatic environment remains a current global research topic. These substances have a strong impact on biological processes, contributing to reductions in the populations of numerous fish and amphibian species. The impact of steroid hormones, especially the third-generation progestogens, on aquatic invertebrates is poorly understood. We aimed to determine whether desogestrel, progestogen of low androgenic activity, affects the reproduction and growth of the following freshwater invertebrates: snails of the species *Melanoides tuberculata* and the planarian *Dugesia* sp. We also tried to estimate the threshold concentrations of this substance at which significant changes in both the behavior and reproductive activity of the studied organisms are observed. In the mesocosm experiment, we performed three treatments with the following different concentrations of desogestrel: control 0 ng/L, medium 10 ng/L, and high 100 ng/L. The high hormone concentration significantly reduced the reproduction of both snails and planarians, despite their different life history strategies, compared to the control. Both planarians and snails showed a significantly lower abundance in the high concentration compared to the 10 ng/L treatment, indicating a threshold concentration > 10 ng/L. The impacts of steroid hormone pollution on aquatic organisms and the need for further research are discussed.

Keywords: *Melanoides tuberculata*; *Dugesia*; desogestrel; reduced reproduction

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1. Introduction

Steroid hormones enter the natural environment in various ways, with scientists most often pointing to biological contaminants as entering waterways through sewage systems and effluents from large factories, industrial plants, and animal farms [1]. Most hormones enter nearby water bodies through sewage systems, creating particularly unfavorable conditions for aquatic plants and animals near sewage outlets, where the highest concentrations are recorded. In addition, hormones enter the natural environment through rainwater, as they are gradually washed out of heavily fertilized crops. It is also important to note the numerous cases of the improper disposal of expired medical waste in many underdeveloped countries in both Europe and South America, contributing to increased hormone concentrations in many rivers in these countries [1,2].

The global market for steroid hormones is an extremely dynamic branch of the pharmaceutical and medical industry. In recent years, there has been an increase in demand for synthetic steroid hormones, contributing to the continued growth of this market seg-

ment. Globally, there has been an increase of approximately 5% year-on-year, while in the Asia–Pacific region, the market value is projected to more than double over the decade [3].

Although all steroid hormones have similar biosynthesis pathways, they differ primarily with regard to the type of receptor with which they interact in the body [4]. After binding to a ligand, a steroid receptor can modulate the expression of target cell genes in the direction of both the activation and repression of transcription. In addition, these receptors regulate the expression of genes involved in stress responses and inflammatory immune processes [5]. Excessive amounts of exogenous hormones in an animal's body disrupt its normal metabolism and endocrine function [6]. The discovery of an ancestral progesterone receptor in 2010 in the rotifer *Brachionus manjavacas* confirmed that steroid hormones have a significant impact on the life and functioning of many aquatic invertebrates. This study demonstrated that progesterone and its receptor retain their function across numerous animal taxa, representing diverse phylogenetic lineages and confirming the ancient origin of steroidal hormonal regulation in both aquatic and terrestrial animals [7].

Previous studies have indicated that the concentration of hormones in deeper water layers and in surface water does not differ significantly to in freshwater environments. Moreover, the quantity of steroid hormones that can dissolve in freshwater is higher than in seawater. It has been reported that in marine environments steroid hormones tend to accumulate in bottom sediments. Consequently, benthic organisms—particularly species living near the seabed or burrowing into marine sediments—are generally the most affected by the adverse effects of these compounds [8]. It is worth noting that the threat posed by high concentrations of steroid hormones is not limited to aquatic invertebrates. A large accumulation of exogenous hormones in the body of a freshwater animal that is captured by a terrestrial vertebrate can cause changes in the predator's body and disrupt its normal hormonal balance.

The aim of this study was to investigate, in a mesocosm laboratory experiment, whether and how the steroid hormone desogestrel affects the behavior and life history strategies, such as the growth and reproductive rate, of the following two invertebrate species belonging to different trophic levels: the *Melanoides tuberculata* deposit feeder snails and predatory planarians of the *Dugesia* genus. The effect of the contamination of third-generation progestogens, such as desogestrel, is poorly documented in aquatic invertebrates [9,10]. Third-generation progestagens include desogestrel (DNG), gestodene (GND), and norgestimate (NGM). Unlike gestodene and norgestimate, desogestrel exhibits low affinity for the progesterone receptor, making it a slightly weaker contraceptive agent that requires a higher minimal effective dose in the human body [11]. Desogestrel has been reported to be a progestogen with low androgenic activity, and is among the weakest of the third-generation steroid hormones in terms of biological activity [6,12].

Therefore, it is essential to investigate the impact of the weakest-acting steroid hormones, which are often overlooked in scientific research in favor of stronger analogs that tend to produce more pronounced and easily observable biological effects. Desogestrel is one of the least studied progestagens and its effects on aquatic animals may indicate a serious problem of steroid hormone contamination of the aquatic environment [13].

We also tried to determine the threshold concentration of desogestrel at which changes in the reproductive activity of the studied invertebrates are observed. We selected invertebrate species because of their specific life history traits. *Melanoides tuberculata* (Müller, 1774) is considered to be a generalist, non-selective (episammic algae and detritus) deposit feeder [14]. This is an invasive snail species that has spread across many countries in North, Central and South America. Its natural range covers vast areas of Asia, from south-western India to south-eastern China. It is also found in the Philippines, Australia, North Africa, Madagascar, and some Central European countries (including Austria and Germany),

where it inhabits geothermal springs or heavily thermally polluted canals [15,16]. This species is characterized by considerable diversity in terms of the shape and color of its shell, which has led to the identification of 27 varieties, which are constantly interbreeding and creating new forms. The snails used in our study belong to variety A. They originate from the central-eastern part of Africa, from Lake Tanganyika in Burundi. This variety is characterized by a dark green shell color with small dark brown spots located at the upper edge of the suture [17]. This snail species is dioecious, but females predominate in many populations and reproduce primarily by parthenogenesis [18]. This is an ovoviviparous gastropod that broods its young in a pouch located in the anterodorsal region [19].

Dugesia sp. planarians are predators inhabiting the bottom zone of freshwater bodies in North America, Europe, Asia, and North Africa. They feed on small crustaceans, insect larvae, nematodes, and snails, including juvenile *Melanoides tuberculata*. *Dugesia* bodies are covered with mucus, which aids gas exchange and makes it difficult for predators to swallow them. Thanks to its regenerative abilities and diverse reproductive strategies (*Dugesia* individuals are hermaphroditic, reproducing both sexually and asexually), they are widely used in scientific research as a model organism [20].

2. Materials and Methods

2.1. The Mesocosm Experiment

The 9 L containers were thoroughly washed to remove any production residues from their surfaces. A separate internal filter with a sponge insert was prepared for each experimental tank. Before placing the filters in the containers, they were pre-rinsed: they were placed in a separate container filled with clean water and run for 2 min to eliminate factory contaminants. The filters were then attached inside the containers using three suction cups and connected to air supply hoses equipped with mechanisms regulating air flow. Each container was filled with 100 mL of water from the same aquarium from which the study invertebrates were obtained, as well as 100 mL of fine-grained gravel, previously thoroughly rinsed under running water.

All 36 containers were then filled with 7 L of water at room temperature. The tanks were left for one week to stabilize the conditions before introducing the invertebrates. The experimental mesocosm containers enabled the partial replication of the natural environmental conditions usually inhabited by the research individuals.

Next, in order to prepare a solution with the appropriate concentration of the test substance, an Ovulan tablet (Adamed Pharma) containing 75 µg of desogestrel was crushed in a mortar and then transferred to a 500 mL beaker. The tablet was dissolved in deionized water with continuous stirring using a glass rod, which allowed a stock solution to be obtained. We chose concentrations of 10 ng/L and 100 ng/L, which correspond to the concentrations of steroid hormones observed in the natural environment 1–500 ng/L [21]. For the medium 10 ng/L and high 100 ng/L concentration treatments, 470 µl and 4700 µl of the stock solution were added to the experimental containers, respectively, using an appropriate automatic pipette. In the control treatment, the desogestrel concentration was 0 ng/L. Finally, the following three treatments with different desogestrel concentrations were performed for the mesocosm experiment: control 0 ng/L, medium 10 ng/L, and high 100 ng/L (12 containers per each experimental treatment).

Before introducing the invertebrates into the respective experimental containers, they were measured in a transparent plastic container (400 mL capacity) placed on graph paper. In the case of planarians *Dugesia* sp., the length and width of the body were determined, while in the case of snails *Melanoides tuberculata*, the length of the shell was measured. Five planarians and two snails were placed in each container (180 and 72 individuals, respectively, in total). Immediately after placing the organisms in the containers, the

planarians were fed with frozen chironomid larvae, which were thawed before feeding, and the snails were fed 'Tropical Breeder' Green Algae Wafers. The food portions were adjusted to the number of individuals, appropriately five chironomid larvae and two plant tablets per container. The organisms were fed twice a week. During each feeding, uneaten food was removed with a pipette to prevent decomposition and the deterioration of the water quality. The collected material was placed on a transparent Petri dish and carefully analyzed to avoid the accidental removal of young specimens. The amount of food throughout the experiment remained constant. Once a month, 3.5 L of water in the containers were replaced with fresh water with the appropriate hormone concentration, to eliminate a potential decrease in its level resulting from the natural decomposition of desogestrel during the experimental period. A comparable methodological approach was employed in a 2014 study that investigated the minimum concentration of progestagens influencing the reproductive processes of the African clawed frog (*Xenopus tropicalis*). Furthermore, that study examined the natural degradation dynamics of steroid hormones using chemical analytical techniques. The findings demonstrated that no measurable degradation of steroid hormones occurred within the 30-day interval between successive water replacements [22].

During the 94 days of our study, the water was changed three times. At the end of the experiment, the study animals were counted and measured. Trophic interactions between the studied species were possible during the mesocosm experiment. The planarians' population could affect the abundance of snails through the consumption of their young. Thus, the abundance of snail offspring was determined based on the total number of living individuals and the empty shells of the offspring, which were noted as 1–1.3 mm long. The reproductive rates (offspring number/initial number of animals) were calculated for each species in different experimental treatments.

2.2. Statistical Analyses

All statistical analyses were performed using PS IMAGO PRO and additionally confirmed in R. Normal distribution was verified using the Shapiro–Wilk test and the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) was used to compare differences in the reproductive effort of the tested animals between treatments with different concentrations of desogestrel. After obtaining a significant test result, a Tukey post hoc test (HSD) was performed, indicating which treatments showed significant differences. A significance level of $\alpha = 0.05$ and a 0.95 confidence interval was used for statistical analysis.

3. Results

3.1. Behavior and Growth of Studied Organisms

The planarians were primarily located near the food aggregations of chironomid larvae in the filter-induced water current. *Melanoides tuberculata*, on the other hand, spent most of their time buried in the sandy substrate. Their mobility was easily noticeable as gully-like tracks in the sand. No differences in the behavior of the studied invertebrates were observed between the treatments with different desogestrel concentrations.

We did not observe statistical differences in the growth rate of the snails and planarians between control and treatments with medium 10 ng/L and high 100 ng/L desogestrel concentration (ANOVA, $F_{2,15} = 0.35$; $p > 0.05$).

3.2. Changes in Reproductive Effort

We noticed a negative effect of desogestrel on the number of offspring organisms in both of the studied organisms: snails *Melanoides tuberculata* and planarians *Dugesia* sp.

Significant differences were observed in the number of the *M. tuberculata* snails between the treatments (ANOVA, $F_{2,15} = 20.5$; $p < 0.00002$). A significantly lower reproductive effort, measured as the snail number, was recorded in the treatment with the desogestrel concentration of 100 ng/L compared to the number of snails in the control and the treatment with the concentration of 10 ng/L (Tukey test, $p < 0.000001$; $p = 0.0003$, respectively, Figure 1). The average *M. tuberculata* reproduction rate was 4.2 in both the control and 10 ng/L treatments. In the treatment with the high 100 ng/L concentration of desogestrel the reproduction rate of *M. tuberculata* was 2.3 times lower, and averaged 1.8.

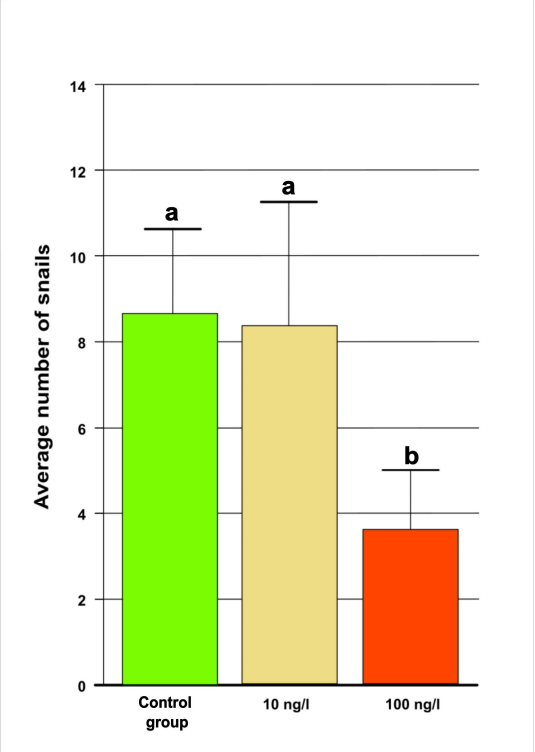

Figure 1. The average number of the *Melanoides tuberculata* snails at different desogestrel concentrations. The error bars represent ±1SE. Different letters indicate statistical differences at a *p*-value of <0.001 (Tukey test, 100 ng/L and control $p < 0.000001$; 100 ng/L and 10 ng/L $p = 0.0003$, respectively).

In the case of *Dugesia* sp., the same effect of desogestrel was observed: a statistically significant difference between the treatments (ANOVA, $F_{2,15} = 35.5$, $p < 0.00000001$). A significant lower increase in the number of planarians was noticeable for the high hormone concentration of 100 ng/L compared to the control 0 ng/L (Tukey's test, $p = 0.00008$), and between the high hormone concentration of 100 ng/L and the medium 10 ng/L concentration treatment (Tukey's test, $p < 0.0000001$, Figure 2).

The average *Dugesia* sp. reproduction rate was two times lower than in *M. tuberculata* snails and amounted to 2.1 and 2.2 in the control and 10 ng/L treatment, respectively. In the treatment with the high 100 ng/L concentration of desogestrel, the reproduction rate of *Dugesia sp.* averaged 0.9.

There was no statistically significant effect of the medium concentration of desogestrel (10 ng/L) on the reproductive rate and the increase in the number of individuals in the experiment, both in the planarians and snails (Tukey's test, $p > 0.05$).

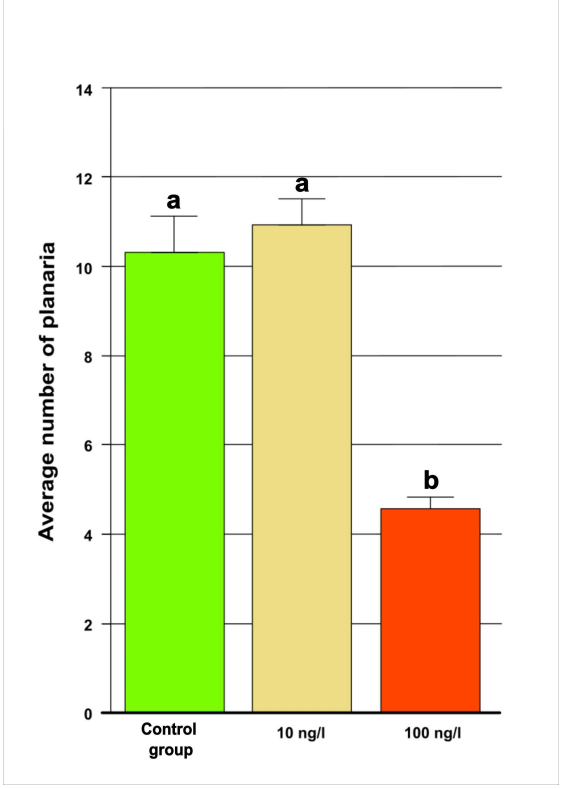

Figure 2. The average number of the *Dugesia* sp. planarians at different desogestrel concentrations. The error bars represent ±1SE. Different letters indicate statistical differences at a *p*-value of <0.0001. (Tukey test, 100 ng/L and control $p = 0.00008$; 100 ng/L and 10 ng/L $p < 0.0000001$, respectively).

4. Discussion

For our mesocosm experiment, we selected two distinct invertebrate organisms, the *Melanoides tuberculata* gastropods and *Dugesia* sp. planarians, differing in their reproductive strategies: dioecy versus hermaphroditism, parthenogenesis vs. sexual, and ovoviviparous vs. oviparous, respectively. They also differed in terms of trophic levels—omnivore vs. predator—and remained in interspecific interactions. Juvenile snails are potential prey for planarians. We indicated that desogestrel reduced the reproduction rate of both these invertebrates. The negative effect was observed only at the concentration of 100 ng/L. As far as we know, these are the first results for these invertebrates and this type of progestogen. The use of a mesocosms system allowed for a better analysis of the natural circulation of steroid hormones in the aquatic environment compared to classic laboratory tests [23]. Importantly, the results obtained under mesocosm conditions may differ from the results of studies conducted on individual species, as the reactions of organisms depend on interspecific interactions and complex trophic relationships [24]. Biomagnification may also occur under such conditions, resulting in organisms at higher trophic levels being exposed to stronger impacts than those at lower levels. In our study, a stronger reduction in the reproduction effort and abundances at the high hormone concentration was observed for *Dugesia* sp. planarians, which fed on juvenile *M. tuberculata* snails. The two-fold lower reproduction rate may indicate, on the one hand, the greater sensitivity of planarians compared to *M. tuberculata*, but, on the other hand, a biomagnification effect is also possible. Each aquatic invertebrate, whether marine or freshwater, is characterized by a specific and relatively stable level of steroid hormones in its body, which it is capable of self-regulating and maintaining within the limits of physiological tolerance. Maintaining this hormonal homeostasis enables the organism to function properly without exhibiting any noticeable morphological or physiological disturbances [25]. It is known that steroid

synthesis and metabolism are among the key processes that can be affected by substances disrupting the normal functioning of the endocrine system. Planarians of the species *Dugesia japonica* and *Dugesia tigrina* have been studied to assess the toxicity of progestagens and glucocorticoids, as well as the effects of these steroid hormones on their regenerative capacities in both central and distal body regions. It was demonstrated that progestagens significantly impaired the regeneration process in flatworms while simultaneously affecting their internal metabolism [26,27]. The crucial role of sex steroids in the functioning of organisms belonging to the phyla Mollusca, Crustacea, and Echinodermata was also confirmed in studies conducted in 2006. In the same research, some responses observed in mussels exposed to crude oil and mixtures of crude oil with alkylphenols were found to be similar to those occurring at the highest progestagen concentrations [28].

Although desogestrel is considered one of the weaker third-generation progestogens, even a small amount in research containers has a negative effect on the physiology and reproduction of both large aquatic vertebrates and smaller aquatic invertebrates. Desogestrel, as a third-generation progestogen, was tested on the *Pimephales promelas* fish at concentrations of 100 ng/L, 1000 ng/L, and 10,000 ng/L. After 21 days, the inhibition of the egg formation process was observed [13]. The threshold concentration of the hormone was 1 µg/l; lower doses did not affect reproduction. The high threshold value may have been due to the short duration of the experiment and the greater complexity of fish organisms compared to invertebrates. At a very high concentration (10,000 ng/L), desogestrel induced the development of secondary sexual characteristics in *Pimephales promelas* within less than a month, which raises questions about the effects of the long-term exposure of aquatic organisms in environments heavily polluted with steroid hormones.

Studies on the impact of steroid hormones on mollusks have been conducted in recent years. In the case of the gastropod *Lymnaea stagnalis*, progestogens at concentrations found in the natural environment (1–500 ng/L) significantly affected their physiology. An increase in heart rate, reduced mobility, and the accelerated embryonic development time were observed. In adult snails, a hormesis effect was also demonstrated in terms of feeding [21]. Experiments with *Placopecten magellanicus* scallops showed that progestogens injections led to irregular sex differentiation, with the predominance of males. High hormone concentrations modulated reproduction similarly to that observed in aquatic vertebrates. Estradiol and testosterone indirectly affected the reproduction rate of laboratory populations, reducing the proportion of females [29]. A similar phenomenon was observed in our study, in which the desogestrel concentration of 100 ng/L caused a reduction in the reproduction rate of the studied gastropod *Melanoides tuberculata*.

The effect of steroid hormones on reproductive strategies in planarians has not yet been thoroughly understood. Recent studies have focused mainly on their impact on regenerative abilities [30]. It has been shown that estradiol accelerates the regeneration of severed body parts, while testosterone inhibits the regeneration process. The presence of receptors that respond to synthetic human sex hormones, modulating the rate of regeneration in *Girardia tigrina*, has been confirmed experimentally. Most of these receptors are located in the distal parts of the planarian body. In turn, research by Tharp et al. [31] suggest that sexually reproducing individuals develop a specific steroid receptor associated with the regulation of sex drive. Once this receptor appears in a population, it is inherited, leading to the consolidation of the trait throughout the genetic line. At the same time, most progestogens and glucocorticoids exhibit toxic effects; both planarian species, *Dugesia japonica* and *Girardia tigrine*, showed a reduction in regenerative capacity as a result of their action, with synthetic hormones being more toxic than natural ones [26,27].

Our results and data from the literature indicate that steroid hormones, both natural and synthetic, significantly modulate regenerative and reproductive processes in aquatic

invertebrates and vertebrates. Their effects are strongly dependent on concentration, species, and exposure time. Progestogens—including the poorly understood desogestrel—exhibit toxicity and lead to reproductive disorders in aquatic invertebrates, such as snails and planarians, as demonstrated in our studies. Incorporating the mesocosm method into the analyses further enables a more realistic representation of the complex interactions between individuals under laboratory conditions. Mesocosms have been reported to be beneficial in providing realistic and controlled studies of aquatic ecosystems that closely resemble those found in nature [32,33]. They enable the study of natural communities and their relationships, such as predator–prey interactions, a characteristic which we exploited in our study.

It is possible to use progestagens as agents for controlling populations of invasive snail species, such as *Melanoides tuberculata*, in artificial water bodies. This type of method has already been successfully applied to invasive terrestrial snails of the species *Theba pisana*, where it proved to be highly effective and, at the same time, environmentally safe. However, the main challenge in the case of aquatic snails remains the environment in which the population control process using steroid hormones takes place—water bodies. Currently, the reduction in invasive aquatic snail populations is mainly carried out using cheaper and more efficient substances than steroid hormones, e.g., copper(II) sulfate or ammonium sulfate [34].

Progestagens may exhibit a cumulative effect that could further enhance their impact on aquatic organisms, making them a potential, though still insufficiently studied, tool for the biological control of invasive snail species. Given the significant ecological and public health importance of managing invasive snail populations, particularly in reducing the spread of parasitic diseases such as schistosomiasis [35], further long-term studies on the cumulative effects of various concentrations of steroid hormones contaminating the natural environment are essential.

5. Conclusions

The conducted study demonstrated that the synthetic steroid hormone desogestrel exerts a measurable inhibitory effect on the reproduction and population growth of freshwater invertebrates, such as *Melanoides tuberculata* and *Dugesia* sp. Despite differences in the life history strategies of both species, and the relatively low biological activity of desogestrel, a clear decline in the reproduction rate was observed at high hormone concentrations comparable to those recorded in natural environments.

The results suggest that desogestrel concentrations exceeding 10 ng/L can represent a threshold above which significant physiological and behavioral disturbances occur. The obtained data highlight the high sensitivity of aquatic organisms to steroid hormone pollution, leading to disruptions in reproductive processes.

Our findings indicate potential ecological risks associated with the presence of steroid compounds in freshwater environments, particularly in the context of the possible use of steroid hormones as a method for controlling populations of invasive aquatic invertebrates. Further research is necessary to elucidate the mechanisms underlying these observed effects and to assess the long-term consequences of hormonal pollution for aquatic ecosystems, as well as for native species that may be unintentionally affected by measures aimed at eradicating invasive species.

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