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Morphometrical Characterization of Green Hydra Symbiosis

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The symbiotic relationship was tested in green hydra species (*Hydra viridissima* Pallas, 1766) using norflurazon and cinoxacin. The goal was to disrupt the balanced symbiotic relationship between hydra and *Chlorella* species with norflurazon and cinoxacin and to monitor changes by using ultrastructural morphometry. Width, area and perimeter of perialgal space were measured using cTEM micrographs. Symbiosomes were ruptured in both test-solutions. Perialgal space area in norflurazon did not change in the 72 hour period, but was severely changed after the recovery period and perialgal space in cinoxacin was enlarged after the treatment. Hydra individuals perished during the recovery phase, though algae survived possibly due to greater protection from the enlarged perialgal space.

Key words: Green hydra, endosymbiosis, norflurazon, cinoxacin, perialgal space, symbiosome.

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Green hydra (*Hydra viridissima* Pallas, 1766) forms an endosymbiotic relationship with green alga of the genus *Chlorella.* Algae, usually up to 20 cells, reside in hydra cells of the gastrodermal, myoepithelial layer (DUNN 1987; HOLSTEIN & EMSCHERMANN 1995). The nature of this endosymbiotic relationship and how both symbionts control it are not well understood (HABETHA & BOSCH 2005). Algal cells are surrounded by perialgal space which is encircled by a plasma membrane called a symbiosome that isolates and protects it from digestion (BURNETT 1973; DOUGLAS 1994). The balanced relationship between symbionts depends on the perialgal space (RAHAT 1992), and both symbiotic partners take part in preserving that balance (CERNICHIARI *et al*. 1969; MCAULEY & SMITH 1982; O'BRIEN 1982; NECKELMANN & MUSCATINE 1983; KESSLER *et al*. 1991).

The herbicide norflurazon and antibiotic cinoxacin were used in this experiment to create unfavourable conditions in which both of the symbionts existence would be threatened. Ultrastructural changes in green hydra symbiosis in an unfavorable environment can be a form of protective mechanism for the endosymbiont in green hydra symbiosis and point towards a certain degree of endosymbiont independence. In this work a quantitative characterization was performed and changes in symbiosomes and perialgal space were expected (KOVAČEVIĆ *et al.* 2005, 2007, 2009), possibly because of their role in maintaining the balanced relationship between the symbionts.

This is a direct continuation of our previous work on hydra symbiosis (KOVAČEVIĆ *et al.* 2007, 2009) and the goal of this research was to test the limits of this endosymbiotic balance by creating unfavourable conditions and to monitor the changes in structure of symbiosomes and perialgal space using ultrastructural morphometry.

Material and Methods

Green hydras were collected in Jarun lake in Zagreb, Croatia. Individuals of hydra attached to submerged plants in lake water were taken and transferred in the laboratory to glass dishes with aerated aquarium water. They were kept at room temperature (21°C) with a photoperiod of 10 hours light, 14 hours dark and fed once per week with *Artemia salina* larvae. Healthy individuals of similar size were chosen for the experiment with no apparent budding.

Three groups of test-organisms were used for this experiment, one group was treated with 117.6 mg/l of aqueous solution of the antibiotic cinoxacin (Lilly Deutschland GmbH, Bad Homburg, Germany) for 72 hrs. The second group was treated with $2x10^{-5}$ mol/l of aqueous solution of the herbicide norflurazon (SAN 9789, Sandoz Ltd., Basel Switzerland; 80% purity) for 72 hrs, after which the individuals were transferred into a glass dish with aerated aquarium water for a further 14 days as a recovery period. The control group of green hydras was kept in aerated aquarium water during the whole experiment. Neither group was fed for the duration of the experiment. There were altogether 30 individuals in each of the three groups. All of the groups were compared to each other.

Standard preparation methods were used for cTEM. Two individuals were taken from both test-groups and after the recovery period as well as from the control group. They were fixed in 2% glutaraldehyde, pH 7.2 buffered with 0.01% sodium-cacodylate and postfixed in 1% osmium-tetroxide buffered with the same buffer. Preparations were then dehydrated, immersed into Spurr resin and cut with a glass knife on the ultramicrotome. Sections were stained with 4% uranyl-acetate and lead-citrate (REYNOLDS 1963). Micrographs were made with electron microscopes Zeiss, EM10A and FEI Morgagni 269D, films Kodak and Imago. Morphometric parameters of width, area and perimeter of the perialgal space were measured using the ISSA program (Vamstec, Zagreb) after the treatments with both norflurazon and cinoxacin and after the recovery period for organisms treated with norflurazon as well as in the control group. Samples of 80 measurements for perialgal space area and perimeter and 50 measurements for perialgal space width were used.

Statistical analysis was done in STATISTICA 12.0 (StatSoft, Inc., USA). Normality of the data was tested by the Shapiro-Wilk's W test. The homogeneity of variance for each variable was tested using Levene's Test. Basic statistical parameters were assessed by the basic statistic method. All the groups were compared to each other. Differences in the mean values of perialgal space width, area and perimeter among the three groups: control (C), norflurazon (Nf) and cinoxacin (Cin) were assessed by one way ANOVA followed by the Newman-Keuls post hoc comparison test. Prior to the tests all the data were normalized by log-transformation. The same tests were applied for the assessment of possible differences among the mean values of width, area and perimeter of the control, the three days treatment with norflurazon (Nf 3 d) and after the 14-day recovery period (Nf 14 d). Statistical significance was set to $P<0.05$.

Results and Discussion

Treatment with norflurazon

The measurable size of perialgal spaces remained the same throughout the three days of treatment with norflurazon, but vastly degraded during the 14-day recovery period (Fig. 3, Table 1). So far, it is known that norflurazon causes mortality, changes in morphology, behavior, locomotion

Table 1

Mean values ($\overline{X}$), standard deviations (SD) and range for width, surface area and perimeter of perialgal space in hydra cells of the control group, hydra cells treated with norflurazon and hydra cells after the recovery period

Parameter	Group					
	Treatment with norflurazon		Control		Recovery period	
	$\overline{X}$±SD	range	$\overline{X}$±SD	range	$\overline{X}$±SD	range
width (μm)	0.37±0.22[c]	0.06-1.07	0.37±0.21[c]	0.10-1.09	0.27±0.19[a,b]	0.06-1.07
area (μm^2)	3.81±2.00[c]	0.07-6.20	3.85±2.22[c]	0.75-9.09	0.83±0.22[a,b]	0.14-2.72
perimeter (μm)	13.75±3.28 [b,c]	6.85-17.71	17.06±5.73[a,c]	7.11-27.42	5.32±1.47[a,b]	2.72-7.92

a-significantly different from norflurazon treated group; b-significantly different from control; c-significantly different from the norflurazon treated group after 14 days of the recovery period ($P<0.05$).

and asexual reproduction, damage to cellular and ultracellular structures and affects overall viability and fitness of green hydra. Transmission electron microscopy revealed antichloroplastal and antimitochondrial effects that result in low viability, degradation and mortality of the organisms. Chloroplasts are described as damaged, swollen, torn apart or degraded and mitochondria as swollen, damaged, changed in shape or with a reduced matrix. The antimitochondrial effect in hydra host cells also occurs, with swollen, damaged or changed mitochondria and a reduced matrix. Fragments of rough ER and Golgi apparatus are present and ribosomes are reduced in number. Concerning the substantial amount of ultrastructural damage, osmyophylic inclusions appear in the vacuolized gastrodermal cells of hydra (KOVAČEVIĆ *et al*. 2007, 2009). After a recovery period, hydras recovered only partially, maintaining permanent damage, but a regular assembly of algae inside the gastrodermal myoepithelial cells of green hydra were noticed (KOVAČEVIĆ *et al*. 2009). Qualitative morphological features include widening of the perialgal space, missing symbiosomes, joining of the existing perialgal spaces and the recovery of green hydra after a period of intoxication.

It is possible that the host cell damages the symbiosomes in an attempt to digest algal cells to enhance its recovery after treatment. Algae respond to this by joining their perialgal spaces and lowering their surface-to-volume ratio and thus reducing the damage done by the host cell. Algae also protect themselves from harmful toxins by widening their perialgal space. Sometimes the widening is brought to the extreme causing complete symbiosome degradation, where it is not detectable any more. This lack of symbiosomes and the altered volume of the perialgal space reveal remarkable changes in the alga-hydra symbiosis (KOVAČEVIĆ *et al*. 2005, 2007, 2009). The widening of perialgal space can be easily detected qualitatively. But in the cases of highly accentuated symbiosome degradation in which the perialgal space was widened to the final exent and symbiosomes were missing completely, it was not possible to perform the morphometry (perimeter of perialgal space), as there was no visible physical border of symbiosomes.

Based on the results of one way ANOVA followed by the Newman-Keuls test, there was no significant difference in the values of mean width of the perialgal space between control samples and those treated for three days with norflurazon (P=0.9297). On the contrary, a significant difference was observed between the control and the recovery group (P=0.00097) as well as between the treated and the recovery group (P=0.00092). The Newman-Keuls test showed no significant difference in the mean area values of the perialgal space of the control and the norflurazon treated group (P=0.95649). However, a significant difference was observed both between the control and the recovery group (P=0.00010) as well as between the treated and the recovery group (P=0.000150). The difference between the perimeter of the control group and both the treated group (P=0.03518) and the recovery samples (P=0.00005) was statistically significant. A significant difference was also observed between the treated and the recovery groups (P=0.000114) (Fig. 3, Table 1).

Treatment with cinoxacin

The size of perialgal space increased slightly after the three-day treatment which confirms previous findings. So far, cinoxacin was shown to exert a strong effect on endosymbiotic alga from green hydra, including changes in color, position, structure and ultrastructure, with some algal ultrastructures completely destroyed. Especially expressed is antichloroplastal and antimitochondrial effect, with irregularities in thylakoid system and swollen mitochondria. After treatment with cinoxacin, the green hydra host suffers severe damage to the gastroderm (KOVAČEVIĆ *et al.* 2005), with mitochondrial membrane structures highly degraded (KALAFATIĆ *et al.* 2016). Qualitatively, wide perialgal space was here noted for the first time (KOVAČEVIĆ *et al.* 2005).

It is possible that algae widen the surrounding perialgal space in order to increase their chances of survival as the host cells attempt to destroy symbiosomes to digest them (DOUGLAS 1994; KOVAČEVIĆ *et al.* 2005, 2007, 2009; NECKELMANN & MUSCATINE 1983). The joining of several algal cells in a single ruptured symbiosome would reduce the surface-to-volume ratio to the surrounding host cell cytoplasm and thus reduce the damage done. Even though treated hydras had larger perialgal spaces than the control group of hydras, the difference in perimeter of both groups is smaller (Fig. 4, Table 2). This indicates that the surface-to-volume ratio is indeed smaller in the experimental group. No data was gathered after the 14-day recovery period from cinoxacin, as symbiosomes were missing completely and it was not possible to measure the paramteres of perialgal spaces as there was no visible border.

It is of yet unknown if the symbiosomes of the survived algae were either damaged by the host hydra cells in an attempt to digest the symbionts to increase their own chances of survival or if the algae themselves could reduce the symbiosomes as there was no longer any danger of digestion after degradation of the hydra host, or if algae simply did not have enough recovery time to form new symbiosomes.

Table 2

Mean values ($\overline{X}$), standard deviations (SD) and range for width, surface area and perimeter of perialgal space in hydra cells of the control group and hydra cells treated with cinoxacin

Parameter	Group			
	Treatment with cinoxacin		Control	
	$\overline{X}$±SD	range	$\overline{X}$±SD	range
width (μm)	0.44±0.31*	0.05-1.53	0.37±0.21	0.10-1.09
area (μm^2)	5.16±3.02*	2.14-13.89	3.85±2.22	0.75-9.09
perimeter (μm)	18.32±4.39	14.30-28.71	17.06±5.73	7.11-27.42

*significantly different at $P<0.05$

Comparison of the groups

When comparing the two treatment groups with each other and with the control in terms of morphological changes of the symbiosome in the process of breakdown of the symbiotic relationship, the perialgal space width, area and perimeter were higher in the cinoxacin treated group compared to the control as well as within the norflurazon treated group, while the norflurazon group treated for 72 hrs showed similar or somewhat lower values compared to the control group (Figs 1-4; Tables 1, 2).

The results of the Neweman-Keuls test confirmed the significant difference between the control and cinoxacin treated groups for width ($P=0.04406$) and area ($P=0.04652$) while there was no significant difference between these two groups in the case of perimeter ($P=0.34172$). When comparing the two treatment groups (both norflurazon and cinoxacin), a significant difference was observed for all three parameters: width ($P=0.04537$), area ($P=0.04954$) and perimeter ($P=0.00120$).

Further tests should be performed to assess which of the two symbionts have control over the formation and destruction of symbiosomes and over the size of perialgal space and how they obtain and maintain it in unfavourable conditions and recovery periods.

Ultrastructural changes were observed in individuals treated both with norflurazon and cinoxacin, in comparison to the control (Fig. 1). The width of perialgal space changed (Fig. 2). Symbiosomes appear either ruptured or completely gone. The damage to symbiosomes causes algal cells to join their perialgal spaces to reduce damage, where up to three algal cells can occupy a single, damaged and conjoined symbiosome and this could lead to the reassembly of the endosymbiosis. Hydra individuals previously treated with norflurazon partially perished during the recovery phase, although algae survive possibly due to greater defense and protection from the enlarged perialgal space (KOVAČEVIĆ *et al.* 2005, 2007, 2009).

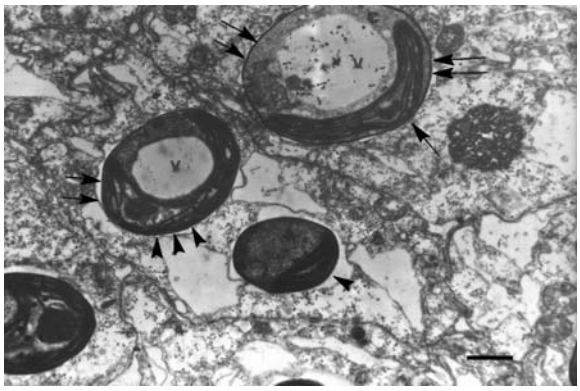


Fig. 1. cTEM of the gastrodermal myoepithelial layer of control green hydra. Host cell (arrow) carrying algal endosymbionts (2 arrows). Perialgal space (arrowhead), symbiosomes (3 arrowheads) and vacuoles (v). Bar=1 μm.

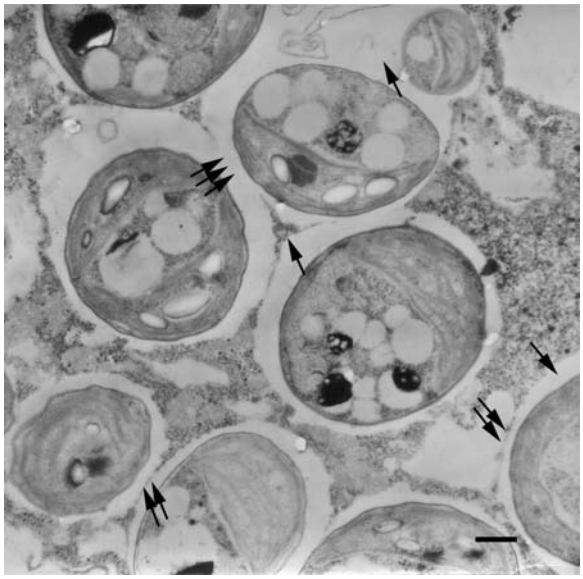
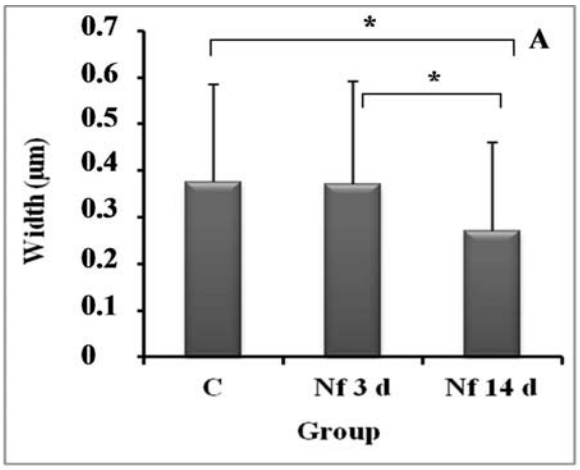
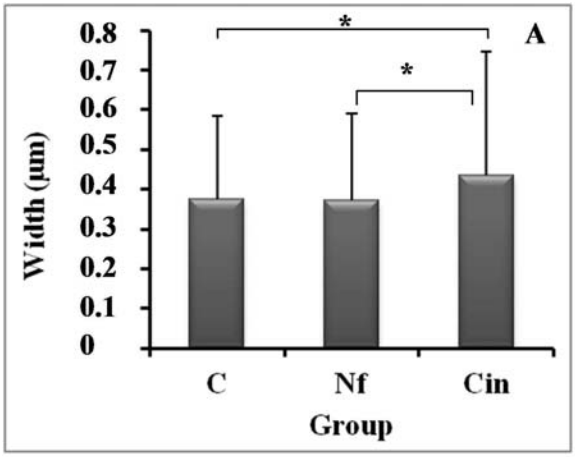
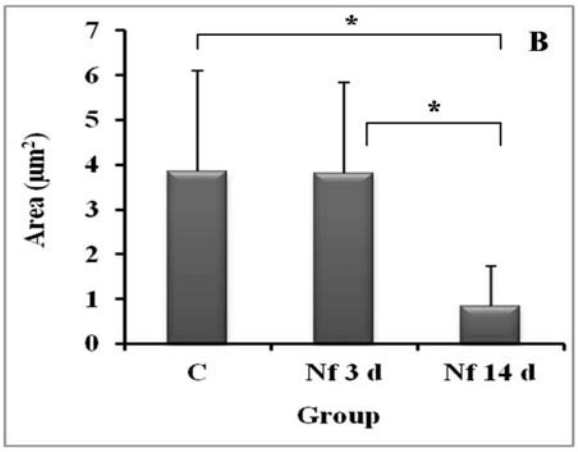
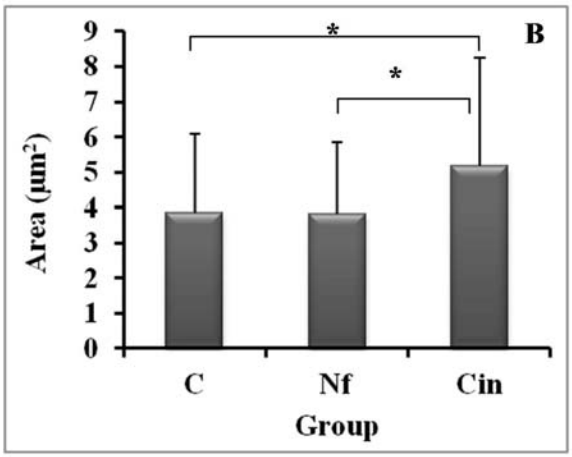


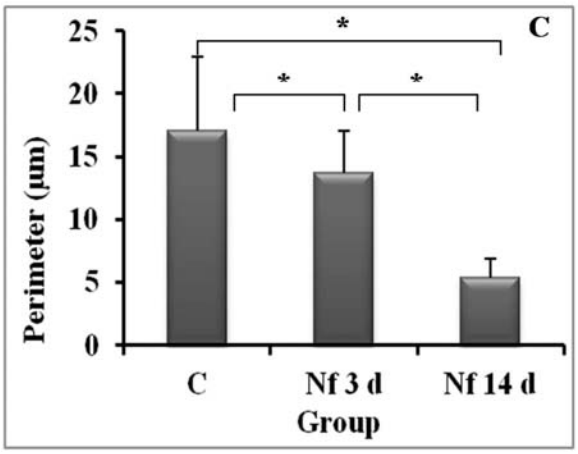
Fig. 2. cTEM of the gastrodermal myoepithelial layer of green hydra treated for 72 hrs with $2x10^{-5}$ mol/l of aqueous solution of norflurazon. Measurable changes in perialgal space (arrow). Reduction and degradation of symbiosomes (2 arrows) and joining of the perialgal spaces (3 arrows). Bar=1 μm.











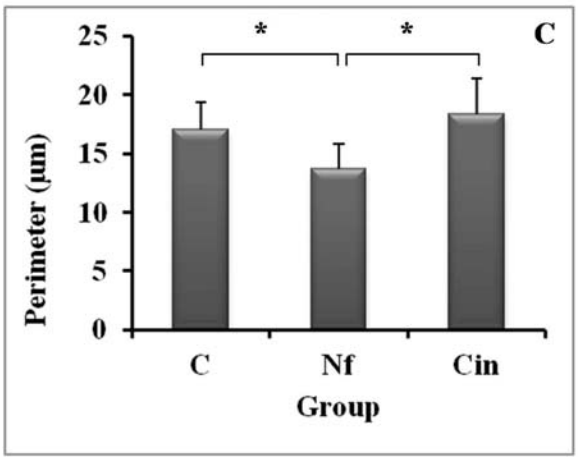


Fig. 3. Mean values and standard deviations for width, area and perimeter of perialgal space in hydra cells of the control group (C), hydra cells treated with norflurazon for three days (Nf 3 d) and hydra cells after the recovery period (Nf 14 d). * – significantly different ($P<0.05$).

Fig. 4. Mean values and standard deviations for width, area and perimeter of perialgal space measured in hydra cells of the control group (C), hydra cells treated with norflurazon (Nf) and in hydra cells treated with cinoxacin (Cin). * – significantly different ($P<0.05$)

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