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Research Article

Humic substances affect physiological condition and sex ratio of swordtail (*Xiphophorus helleri* Heckel)

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Abstract. Exposure of young swordtail (*Xiphophorus helleri*) to increasing concentrations of synthetic humic substances (HS1500) significantly effected the physiological condition and slightly effected the sex ratio of the fish. Any exposure enhanced the body mass development of the swordtail compared to the control. No dose-dependent effect of HS on growth was detectable. After a two-week period of stressful handling, the HS-exposed fish recovered quickly whereas growth stagnated in the control fish. The examination of gills, fins, and skins revealed no infestation with ectoparasites. We assume that the growth promoting effect of HS1500 was due to an overall stimulation in metabolism. Furthermore, the sex ratio of fish exposed to HS1500 for 21 weeks slightly shifted in favor of females in a dose-dependent manner. Although the mode of action is still obscure, there is a probability that alkylphenol structures in HS1500 may be responsible for this weak feminization.

Key words. Humic substances; fish growth; fish condition; sex ratio; feminization.

Introduction

Humic substances (HS) comprise the majority of dead organic matter and exceed the carbon levels in all living organisms by more than one order of magnitude (Steinberg and Münster, 1985; Wetzel, 2001). HS are polydispersed polyelectrolytes, and, as such, they share a characteristically high degree of molecular irregularity and heterogeneity (Orlov, 1995). As a consequence, no two identical HS molecules exist in water or soil, implying that one cannot give a general structural formula for HS. Usually the HS molecule must be described by statistical features: ratios and variations of the various structural entities (Davies and Ghabbour, 1998). As a consequence of their molecular irregularity and heterogeneity, HS were thought to be almost inert or refractory because repetitive structures are rare that can be attacked by, for instance, microorganisms. Hence, HS were considered not to take part in metabolic processes of freshwater ecosystems or directly affect freshwater organisms. If there were effects on organisms exposed to HS, they were assumed to be of an indirect nature via the modulation of bioavailability or release of inorganic and organic trace nutrients (Münster et al., 1999), or partial damping of exo-enzyme activities (Münster et al., 1992, 1999; Boavida and Wetzel, 1998).

The ecochemical relevance of HS is mostly discussed with respect to their capability to bind or integrate pollutants like organic xenobiotics and heavy metals, consequently decreasing the bioavailability and toxicity of these pollutants (Haitzer et al., 1998; Meinelt et al., 2001). However, the same functional groups also may interact directly with biological systems such as particular membranes (Tranvik, 1990; Vigneault et al., 2000). As net effects, modulation of the survival and growth of inver-

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tebrates (Petersen and Persson, 1987) and plants (Chen et al., 1994), enzyme activities (Pflugmacher et al., in press; Wiegand et al., in press), and antimicrobial and antimycotic activity (Bärlocher, 1992) may occur. In addition, photochemical reactions can initiate a chain reaction, leading to reactive oxygen species (ROS) such as $^1O_2$, O_2^- and H_2O_2 (Frimmel, 1998) that are able to provoke oxidative damage to cell structures (membranes).

There are ambiguous reports of direct impacts of HS on fish. Non-specific energy consuming reactions in the biotransformation system, induction of chaperons (hsp70) (Wiegand et al., in press), as well as increased survival rates and increased physiological condition (Steinberg et al., 2003) have been reported in fish. This obvious contradiction in effects deserves resolution. Additionally, an unexpected result has been the modulation of offspring numbers in the nematode *Caenorhabditis elegans* (Höss et al., 2001; Steinberg et al., 2002). An off-spring modulating effect could be observed mainly in an increase in offspring numbers. Since estrogen receptors have been discovered recently in this invertebrate, the observed effect could be estrogen related (Hood et al., 2000). Höss et al. (2001) and Steinberg et al. (2002) used HS and NOM (Natural Organic Matter) isolates from the environment as exposure materials. Hence, a non-point contamination with man-made endocrine disrupting chemicals cannot be excluded.

To exclude effects other than those directly from the HS, we applied a man-made HS and addressed the following questions:

1. What is the net-effect of synthetic HS on the physiological condition of fish?
2. Is the pseudo-hormonal effect, as observed with the nematode *C. elegans* also detectable in fish.

The sex ratio of lower vertebrates such as fish and amphibians is known to be influenced by environmental factors like temperature, endocrine disrupting man-made chemicals, population density, and sex ratio of the population. Since the swordtail (*Xiphophorus helleri* Heckel) is one of the best documented fish species with respect to external sex determining factors we used the swordtail in our experiments.

Materials and methods

Prior to the experiment, new born swordtails were fed nauplius larvae of *Artemia salina* for two weeks. Subsequently, 50 juveniles were placed in each of four 20 L glass aquaria. The aquaria were equipped with aeration and a foam filter. Water for aquaria was prepared from deionized water by adding 103 mg/L $NaHCO_3$, 268 mg/L $CaCl_2 \times 2H_2O$ and 100 mg/L sea salt (Tropic Marin, Biener GmbH, Wartenberg, Germany) and kept at 26 °C.

Over a period of 21 weeks, the fish were exposed to different concentrations of the synthetic humic substance HS1500 (Sophar Pharma GmbH, Mannheim, Germany). HS1500 was produced by controlled autoxidation of hydrochinone, and characterized as a humic substance (HS) with a median particle mass of 1500 Da (Schulten et al., 1991; Schulten, 1995). HS1500 is composed of 35–39% carbon, 3% hydrogen, and 38–42% oxygen (Seubert et al., 1987). It contains no heterogeneous peripheral structures or toxic components such as heavy metal ions.

The following test groups were established: control, 0 mg/L DOC; group 1, 5 mg/L DOC HS1500; group 2, 30 mg/L DOC HS1500; group 3, 180 mg/L DOC HS1500. Concentrations of 5 mg/L C are normal for environmental conditions in freshwater, and concentrations of 30 mg/L C can easily be found in bog waters. In addition to HS concentrations occurring under natural conditions, we selected the highest concentration of 180 mg/L C to ensure that any possible effects of HS on fish could be detected.

The pH over the investigation period was 7.9 ± 0.1 (control), 7.7 ± 0.1 (5 mg/L-group), 7.5 ± 0.1 (30 mg/L-group), and 7.4 ± 0.15 (180 mg/L-group). Fish were fed TetraMin flakes (Tetra, Melle, Germany) 5 × per day, 6% of their total mass over the first 12 weeks. In the following 9 weeks, the amount of food was reduced stepwise to 3% until the end of the experiment. All fish were weighed once per week and the necessary amount of food was re-calculated on the basis of the stock mass. The filters were cleaned and 5 liters of test water were replaced each day by water prepared and supplemented with HS as described above. Dead fish were removed and counted.

Fish from all groups were netted daily for a period of two weeks to investigate the influence of HS1500 on stress.

At the end of the experiment, fish were killed with benzocaine-solution (200 mg/L). Length and mass of each fish, as parameters of the physiological condition, were determined separately for males and females because the growth of the two sexes is different in swordtails. The corpulence-(condition-)factor was calculated as given by Schäperclaus (1990):

$$k = \frac{100^* p}{L^3}$$

(were k = corpulence factor, p = mass, and L = length).

Skin, fins, and gills of each fish were examined microscopically for ectoparasites.

Statistics

The mass and length of fishes in control and the three HS-groups were compared by ANOVA. Because of different sex-specific growth rates, data from males and females

were analyzed separately. ANOVA tested the null hypothesis "all groups are equal" against the alternative "at least one group is different". For a more detailed analysis, Scheffe's post-hoc test was used to detect homogeneous subgroups. Analysis was performed with SPSS at a significance level of 5% ($p = 0.05$).

Results

The mean body mass of *X. helleri* in the different groups is shown in Figure 1. Within the 3 HS-groups, no dose-dependent effect of HS on growth was detectable. In all groups, the initially continuous increase in body mass stagnated between week 9 and 11 when fish were stressed by daily netting. Following the stress treatment, fish in all HS-exposed groups continued their steady growth for 2 weeks, even at an increased growth rate. In contrast, the growth of control fish continued at a rate comparable to the beginning of the experiment, and growth increased only slightly following week 12. The body mass of control fish remained similar to pre-stress levels at the end of the experiment.

The survival of control fish and fish of the HS-exposed groups was similar (Control = 80%, group 1 = 88%, group 2 = 82%, group 3 = 88%). At the end of the experiment, both sexes of *X. helleri* in the control group were significantly shorter and lighter compared to the HS-exposed groups (Figs. 2, 3). Scheffe's-test revealed no significant differences in the length and weight of males and females between the three groups of fish exposed to HS (Table 1). However, a slight and significant dose-de-

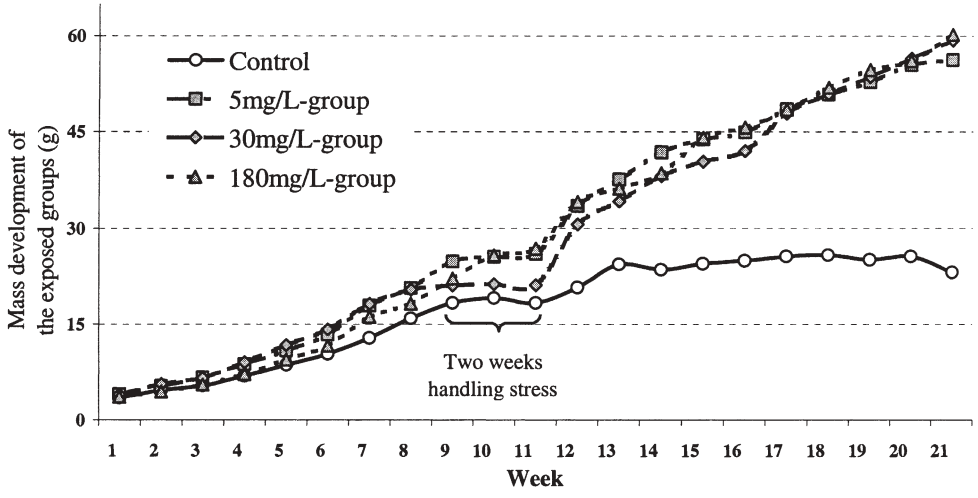

Figure 1. Mean individual mass of HS-exposed *X. helleri.*

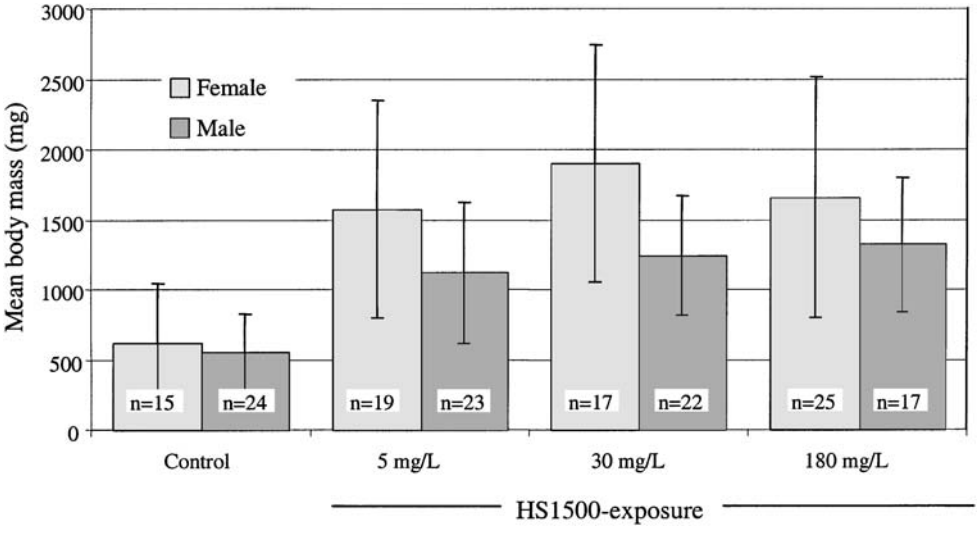

Figure 2. Mean body mass of male and female *X. helleri* after a 21-week exposure to HS1500 at different concentrations.

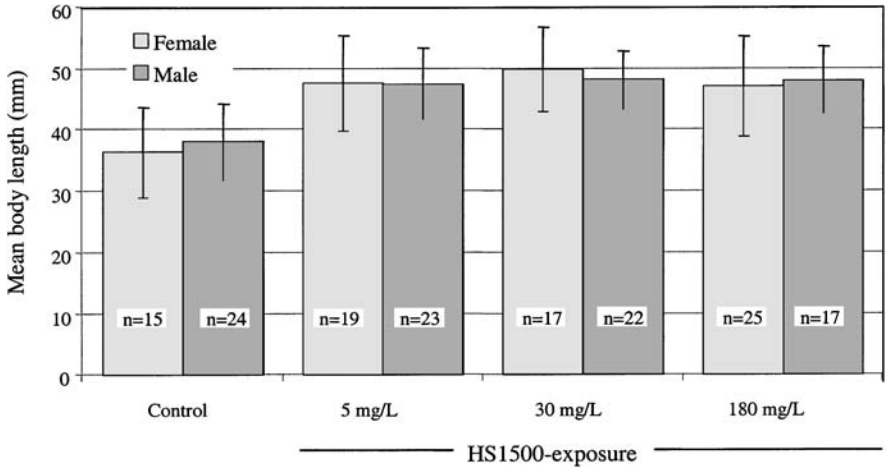

Figure 3. Length of male and female *X. helleri* after a 21-week exposure to HS1500 at different concentrations.

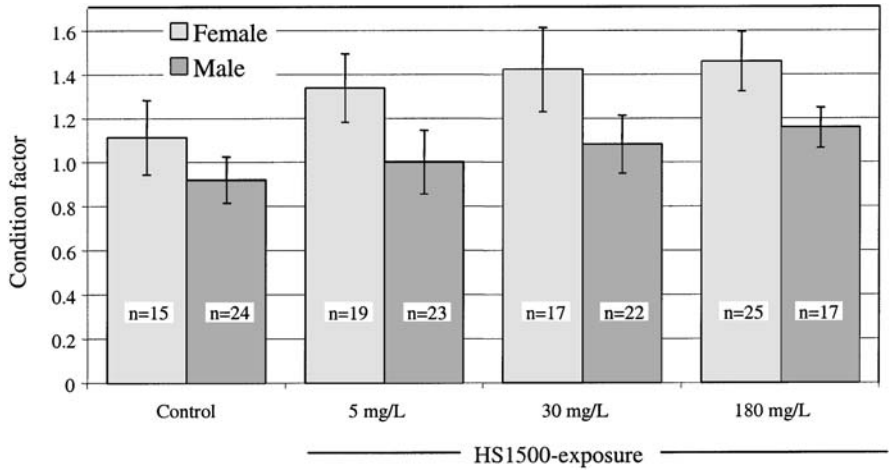

Figure 4. Corpulence (condition) factor of male and female *X. helleri* after a 21-week exposure to HS1500 at different concentrations.

Table 1. Pairwise comparison of means of the test groups (Significance level, p = 0.05). ANOVA (Scheffe'-test).

	Female length growth			Male length growth			Female mass growth			Male mass growth		
	group 1	group 2	group 3	group 1	group 2	group 3	group 1	group 2	group 3	group 1	group 2	group 3
Control	0.007	0.000	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000
group 1		0.658	0.989		0.809	0.545		0.868	0.996		0.988	0.993
group 2			0.798			0.961			0.732			1.000

Control: 0 mg/L DOC HS1500; group 1 : 5 mg/L DOC; group 2 : 30 mg/L DOC; group 3 : 180 mg/L DOC.

Table 2. Pairwise comparison of means of the test groups.

	Female condition factors			Male condition factors		
	group 1	group 2	group 3	group 1	group 2	group 3
Control	0.002	0.000	0.000	0.161	0.000	0.000
group 1		0.504	0.125		0.198	0.002
group 2			0.913			0.296

(for further details, see Table 1).

pendent increase in the corpulence factor was detected for males (Fig. 4, Table 2). The percentage of female fish increased in the HS-exposed groups, and the proportion of females was positively correlated with HS1500 concentration (Fig. 5). Approximately 80 % of the decrease in males and 60 % of the increase in females could be statistically attributed to HS-exposure. The proportion of individuals with un-identifiable sex was below 5 %. As a

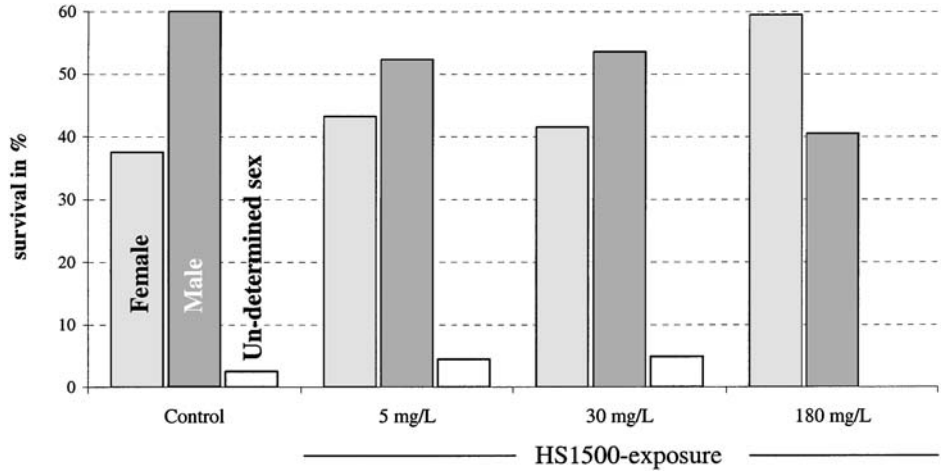

Figure 5. Sex ratio of *X. helleri* after a 21-week exposure to HS1500 at different concentrations.

result, HS-exposure might be considered and discussed as one factor in the change in the sex ratio.

Examination of gills, fins, and skin revealed no infestation with ectoparasites in any of the groups.

Discussion

Exposure of young *X. helleri* to HS1500 showed a favorable effect on the condition of fish, resulting in a slightly increased growth rate in 2 to 9 week old fish. Interestingly, the capability of fish to compensate for stress was increased by HS1500 exposure. The influence of moderate stress by netting resulted in an inhibition of growth in all fish. However, after the end of the stressful treatment, HS-exposed fish were able to compensate for the delayed growth and constantly grew until the end of the experiment at week 21. In contrast, control fish hardly recovered from the stress. They were unable to compensate the stress-induced delay in growth, and, following week 12, only a slight increase in mass occurred. Consequently, all fish in the HS-exposed groups were significantly longer and heavier than control fish at the end of the experiment.

It is known that steady stress, caused by handling or transport, can lead to an outbreak in disease. Growth and reproduction are impaired and the immune system is suppressed (Wendelaar Bonga, 1997). These effects of stress can explain the detrimental effect of handling stress on the growth of control fish. In contrast, HS-exposed fish were able to compensate for stress very quickly. Positive effects of HS in peloids have also been reported in the health of mammals (Ziechmann, 1996). It has been shown that HS have disinfectant and wound healing effects on skin lesions, as well as bacteriostatic, virostatic, and astringent and antiphlogistic effects. Further, the physical properties of fibrous proteins are improved and the activity of (human) granulocytes is increased by HS (Riede et al., 1991, 1992). In previous studies, Schreckenbach (1990) and Schreckenbach et al. (1991, 1994, 1996) demonstrated that HS are able to influence the health and physical condition of fish. An important effect of HS on the health of fish is achieved by an enhanced stability of the mucosa, the first fundamental barrier against pathogens. Schreckenbach et al. (1996) showed that the amount of mucosa cells increased in HS-exposed fish.

Studying HS1500-exposed fish, Schreckenbach et al. (1996) summarize the net health-promoting effects of HS as follows:

1. general improvement of physiological processes,
2. stimulation of immune response,
3. impairment of pathogens by reducing their capacity to adhere and penetrate.

By activating the granulocytes, the non-specific defense of fish is activated and damaged cells will increasingly be incorporated. These positive effects of HS can explain the enhanced growth of HS-exposed fish. First, the metabolism as well as the defense system are supported by HS; namely, the mucosa barrier against invading pathogens is strengthened and the phagocytic activity of defense cells might be enhanced. Second, the viability and, therefore, the pathogenicity of pathogens is reduced by HS. Even if no parasitic infections could be detected in our study, a permanent threat of the fishes by ubiquitous, facultative pathogenic bacteria must be assumed. Wiegand et al. (in press) showed that exposure to HS (as NOM, natural or artificial HS) provoked non-specific defense reactions in invertebrates and fish such as chaperon (hsp70) expression or modulation (mainly increases) of biotransformation enzyme activity. These reactions consume energy, but stimulate the metabolism. The stimulation of metabolism and defense mechanisms, in combination with a reduced loss of energy for pathogen defense may result finally in a higher net growth of fish.

HS containing hydrochinone, like HS1500 used in this experiment, are known to be highly reactive (Ziechmann, 1996). Although the condition factor of male fish was slightly increased at higher HS-concentrations, this was not observed for females and no dose-dependent decrease in growth-promoting properties of HS1500 was detectable at the lowest concentration used in this experiment (5 mg/L). Since the growth of *X. helleri* was similar in all HS-exposed groups (5, 30, 180 mg/L DOC), one can conclude that the condition-promoting effects of HS1500 are induced even at low HS-concentrations. This finding opens a promising area of environmently friendly application for HS in fish culture.

In addition to the described positive effects on growth and capability to compensate for stress, exposure to HS1500 resulted in a slight dose-dependent feminization in *X. helleri*. For this species, Rubin (1985) demonstrated a significant androgenic effect of pH 6.2 compared to pH 7.8. In contrast, in our study the percentage of males even decreased in groups maintained in water supplemented by HS that resulted in a slight decrease in pH. Therefore, the observed effects can be attributed to HS and not to low pH. Slight antiandrogenic-like effects of HS1500 on rats, as indicated by reduction in sperm numbers and testis-atrophy, are reported by Kühnert et al. (1995). Although it is known that phenotypic sex differentiation of several fish species is influenced by environmental factors like pH or water temperature, and sex change is a well-known phenomenon in *Xiphophorus* spp., up to now nothing is known about the influence of HS on sex differentiation. However, some hypotheses can be discussed.

From studies with a variety of alkylphenols, it is well known that these xenobiotics may cause feminization in young male fish and amphibians via an extrogenic mode of action (Gimeno et al., 1997; Nakamura et al., 2002; Schwaiger et al., 2002). Furthermore, since alkylphenols are major constituents of terrestrial and aquatic HS (Schulten, 1999), and since HS with molecular masses of several hundred Da can be taken up by aquatic organisms (Steinberg et al., 2003), an estrogenic mode of action may apply in the sexual differentiation of *X. helleri* – provided that the alkylphenol of the HS can interact with the estrogene receptor. It will be interesting to examine if this effect is only restricted to *X. helleri* or can be found in other fish or amphibian species. Further studies are necessary to confirm the molecular mechanisms that this effect is based on.

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