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Endocrine Effects in *Gammarus fossarum* (Amphipoda): Influence of Wastewater Effluents, Temporal Variability, and Spatial Aspects on Natural Populations

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Abstract. In a field study, individuals of autochthounous populations of the amphipod *Gammarus fossarum* were examined for their maturity status, oocyte development, and biochemical parameters associated with their reproductive cycle. Variability in these parameters was related to abiotic exposure parameters varying in accordance to wastewater discharges, stream identity, and time. Patterns of all investigated parameters showed predominantly seasonal rather than spatial influence for both exposure and biologic effects. Single selected-effect parameters, however—such as the maturity index, late vitellogenic oocyte size and atresia, and the hsp90 level—responded to a sewage treatment plant discharge showing an estrogenic potential and also correlated significantly with the concentration of potential xenoestrogens at the different locations.

Endocrine disruption in wildlife has become a common object of research in recent years. For many substances, an endocrine disrupting effect has been demonstrated, and for even more substances this capacity is presumed. At the end of the 1970s, failure in reproduction of ospreys were one of the first phenomena linked to xenoestrogens (Spitzer 1978); other studies–such as those by Mason *et al*. (1986), Fry *et al*. (1987), or Guillette *et al*. (1994)–followed. To date, research is still mainly focused on vertebrate species, and little is known about the underlying mechanisms outside this subphylum with the exception of prosobranch snails (Oehlmann *et al*. 1996).

In other invertebrate phyla, recent studies have shown effects of potential endocrine disruptors on reproductive parameters or sex ratios (Pascoe *et al*. 2002; Kirkebride-Smith *et al*. 2001; Watts *et al*. 2001). In these studies, mostly laboratory tests using static or flow-through systems have been applied (Duft *et al*. 2003; Watts *et al*. 2002; Andersen *et al*. 2001). In this context, a strong impact of the artificial estrogen, 17α-ethinylestradiol, has been described on the sex ratio in cultures of freshwater crustaceans of the genus *Gammarus* (Watts *et al*. 2002). Gammarids are prominent elements of the limnofauna of the northern hemisphere and widely abundant in streams. Because their habitats are potentially influenced by estrogen hormone residues introduced by way of sewage treatment plant effluents, a disruption of sex ratios and reproductive cycles, or the regulation of the endocrine system, would be crucial for the fate of established natural populations. However, population size and structure may also vary considerably under natural conditions, and it is unclear whether other environmental parameters of nonchemical character could even have a larger influence on reproductive parameters in *Gammarus* spp. than potential estrogens.

To investigate the impact of both natural environmental variability and artificially introduced household wastewaters, we used the following study design. We addressed spatial variability by choosing two small streams, one in Southwest Germany and the other in Eastern Germany; we addressed seasonal aspects by sampling in spring and autumn; and we also considered variability of two sequential years. In contrast, the rather continuous parameter in this study was the influence of each sewage plant on both streams in whose effluents potential endocrine disruptors were present. Using upstream and downstream sampling sites at both streams, the influence of potential sources of endocrine-disrupting chemicals was tested under field conditions. At the beginning of our study, no information on the mechanisms of the endocrine action of xenobiotics in *Gammarus* spp. was available. Therefore, the selection of end points, presumably indicating effects on the reproductive system, was biased. Because Tan-Fermin and Pudadera (1989) have described the female maturation cycle in crustaceans, it was reasonable to select histologic end points of oocyte maturation as measures of reproductive cycle integrity. At the biochemical level, Schirling *et al*. (2004) found a strong covariation of the level of the protein hsp90 with the reproductive cycle. In early stages of oocyte development, the hsp90 level of individuals was lower than in specimens with mature eggs by a factor of three. In vertebrates, it is known that hsp90 is of crucial importance for steroid receptor interactions and modulates sex

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hormone signal transduction (Pratt and Toft 1997). The existence of an equally complex system has not been proven for invertebrates. However, the recent discovery of estrogen receptors in invertebrate taxa, both in the deuterostome and protostome clade (de Waal *et al*. 1982; di Cosmo *et al*. 2002) indicate that steroid-binding proteins and therefore mechanisms associated with the signal transduction process are phylogenetically very old (Thornton *et al*. 2003). We selected hsp90 as a potential biochemical end point, particular in view of possible uncoupling effects, although it is well known that hsp90, like all stress proteins, also responds to stressors that do not target the endocrine system. As a measure of such non–endocrine-disruptor stressor effects, we therefore included the well-established general stress marker hsp70 in our study, predominantly as an ''unspecific stress effect control'' for hsp90.

The aim of this study was to answer (1) whether we can trace possible endocrine effects in gammarids in a highly variable environment; (2) whether biomarker responses occur predominantly downstream of sewage plant discharges; and (3) whether variability in the selected biomarkers can be attributed to the influence of endocrine-disrupting potentials or, alternatively, to environmental parameters varying in space and time.

Material and Methods

Field Exposure

In 2000 and 2001, adult male and female gammarids (*Gammarus fossarum* [Koch 1835]) from two streams were collected for this study. One of the streams, the Lockwitzbach near Dresden, East Germany, has a lower impact of sewage treatment plant effluent, whereas the Körsch, near Stuttgart, Southwest Germany, comprises a mixture of pollutants caused by intensive agriculture and a series of sewage treatment plants (Adam *et al*. 2001). To examine environmental effects on *G. fossarum* upstream and downstream of the effluent of the most upstream sewage treatment plant, two sampling sites were established in each stream. The four sites were referred to as Lu (Lockwitzbach upstream), Ld (Lockwitzbach downstream), Ku (Körsch upstream), and Kd (Körsch downstream). At both streams, the upstream and downstream sites were approximately 6 km apart. The content of all four sampling sites, plus the respective waste water effluent, were characterized by chemical analyses for a series of steroids, phenols, phthalates, pesticides, and their metabolites every month between April and October 2001 (results presented in Jungmann *et al*. 2004b). Furthermore, limnochemical parameters were analyzed in parallel to the animal samplings, and, additionally, every 4 weeks between April and October in both 2000 and 2001 (Table 1). In both years of the investigation, two samplings took place, one in April and one toward the end of the reproductive period of *G. fossarum* in October. At each site, gammarids were collected by kick sampling and their body length measured and separated into two groups: medium (M) size ($6 \leq x \leq 9$ mm) and large (L) size ($x > 9$ mm). The number of replicates differed according to site, sampling time, and investigated parameter because of the abundance of gammarids, percentage of male animals in the sample, etc. (Table 2).

Stress Protein Analysis

For the stress protein (hsp90 and hsp70) analysis, the gammarids were shock frozen in liquid nitrogen on-site. The animals were individually homogenized in extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, and 2% protease inhibitor [Sigma P8340, pH 7.5]), the volume of which was adjusted to the individual's body weight and the homogenate subsequently centrifuged (for 12 minutes at 20,000 g at 4°C). Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (40 μg total protein per lane) were subjected to sodium dodecyl sulphate–polyacrylamide gel (12% acrylamid-bisacrylamid) for 20 minutes at 80 V and for 120 minutes at 120 V in duplicate (samples for hsp90 and hsp70 analysis were run on separate gels). Protein was transferred to nitrocellulose by way of semidry blotting, and the filter was blocked for 2 hours in 50% horse serum in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5). After washing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, Hamburg, Germany, dilution 1:5,000 in 10% horse serum/TBS or mouse anti-water mold hsp90; StressGen Victoria, Canada, dilution 1:800) was added and incubated at room temperature overnight. After repeated washing in TBS for 5 minutes, the nitrocellulose filters were incubated in the second antibody (peroxidase-conjugated goat anti-mouse immunoglobulin G; Dianova, dilution 1:1,000 in 10% horse serum/TBS) at room temperature for 2 hours. After repeated washing in TBS for 5 minutes, the antibody complex was detected by 1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The grey scale values of the Western blot protein bands were quantified using a densitometric image analysis system (Herolab E.A.S.Y., Germany) and related to an hsp90 or, respectively, an hsp70 standard run in parallel on each gel.

In the primary experimental set-up, only hsp90 measurements were included, which explains the lack of data on hsp70 for the first sampling. To assess the general stress status in the gammarids, however, we decided also to include hsp70 analysis from fall 2000 onward.

Histologic Analysis

On site, the gammarids were decapitated and fixed in 2% glutardialdehyde and dissolved in 0.005 M cacodylate buffer. Before embedding, the gammarid samples were decalcified in 5% trichloroacetic acid for 2 days. The samples were dehydrated in a graded series of ethanol and finally embedded in Technovit (Heraeus Kulzer, Germany). Then the animals were sectioned as follows: per individual, 8 series each of 16 sagittal sections (4-μm thickness each) were cut on a Reichert Jung 2050 microtome. The arrangements of the 8-section series depended on the individual width of each specimen, thus assuring a complete overview of the histology of each individual. Sections were stained with azur–methylene blue according to Richardson *et al*. (1960), and examined using a light microscope (Zeiss, Axioscop 2).

The female animals were classified based on the maturity status of their gonads. This was accomplished by examining the maturity of the oocytes. In this study, five different stages of oocytes were distinguished on the basis of the work of Tan-Fermin and Pudadera (1989) (Fig. 1): (1) previtellogenic stage (P) = small basophilic cells with a large nucleus, but lacking vesicles; (2) early vitellogenic stage (E) = small basophilic cells with few vesicles in the cytoplasm and occurrence of perinuclear heterochromatin; (3) late vitellogenic stage (L) = larger, cubic cells with large vacuoles of lipids or proteins, and follicle cells surround the outer membrane of these oocytes; (4) mature stage (M) = last stage of the oocytes before being released to the marsupium; characteristic egg membrane surrounds the cell, and the ellipsoidal nucleus is located next to the membrane; and (5) spent stage (S) = only oocytes which are not released into the marsupium belong to this stage; they become atretic and undergo lysis. In addition, a sixth ''stage'' of oocyte development at the beginning of the

Table 1. Physicochemical parameters at the sampling sites in 2000 and 2001

Parameter		Lu	Ld	Ku	Kd
Oxygen (mgl^{-1})	Median	10,1	9,8	9,3	9,5
	25% to 75%	9,7/10,9	9,3/10,5	8,8/10,6	9,1/10,3
	min/max	9,0/11,8	8,2/12,0	6,9/13,7	7,9/11,8
pH	Median	7,8	7,7	8,2	8,2
	25-75%	7,6/7,9	7,4/7,8	8,1/8,3	8,1/8,3
	min/max	6,4/8,0	0,0/8,5	7,7/8,9	8,0/8,6
Temperature (°C)	Median	12,6	13,1	13,0	14,8
	25-75%	11,0/14,4	11,6/15,0	11,8/14,4	13,3/16,0
	min/max	6,0/16,1	7,0/15,8	8,0/16,3	10,6/17,4
Conductivity [μScm^{-1}]	Median	355	589	917	838
	25-75%	330/374	502/629	880/947	800/909
	min/max	267/467	435/908	711/1725	533/960
Nitrate-N [mgl^{-1}]	Median	7,0	9,8	1,9	6,9
	25-75%	6,0/8,6	7,3/11,1	1,5/2,3	4,9/9,4
	min/max	4,1/11,7	4,6/14,2	1,1/8,4	2,8/11,1
Nitrite-N [mgl^{-1}]	Median	0,01	0,03	0,01	0,02
	25-75%	0,01/0,03	0,01/0,05	0,01/0,02	0,01/0,04
	min/max	0,00/0,05	0,01/0,13	0,00/0,15	0,01/0,09
Ammonium-N [mgl^{-1}]	Median	0,02	0,07	0,04	0,02
	25-75%	0,02/0,10	0,03/0,10	0,01/0,07	0,01/0,09
	min/max	0,00/0,10	0,00/0,75	0,00/0,20	0,00/0,34
Ammonia-N [mgl^{-1}]	Median	0,00	0,00	0,00	0,00
	25-75%	0,00/0,00	0,00/0,00	0,00/0,00	0,00/0,00
	min/max	0,00/0,00	0,00/0,20	0,00/0,01	0,00/0,01
SRP-P [mgl^{-1}]	Median	0,20	0,31	0,52	0,94
	25-75%	0,19/0,23	0,26/0,40	0,21/0,90	0,45/2,13
	min/max	0,10/0,30	0,15/0,79	0,11/11,3	0,34/3,9
Silicon [mgl^{-1}]	Median	5,0	6,2	nd	nd
	25-75%	4,4/5,3	5,3/6,6	nd/nd	nd/nd
	min/max	3,0/6,2	4,7/7,8	nd/nd	nd/nd
total H [°dH]	Median	9	15	13	11
	25-75%	8/11	13/16	11/15	8/14
	min/max	4/16	7/19	8/18	5/17
carbon H [°dH]	Median	4	8	12	11
	25-75%	3/4	7/10	10/14	8/12
	min/max	1/9	5/13	8/18	8/14
non carbon H [°dH]	Median	5	6	1	1
	25-75%	4/7	5/7	0/2	−2/3
	min/max	−5/12	−5/11	−5/3	−4/5

H = hardness; Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitbach upstream; SR = Psoluble reactive phosphate.

Table 2. Number of replicates per sampling for histologic and biochemical investigations

	Histology				Hsp90 and Hsp70			
Month and Year	Lu	Ld	Ku	Kd	Lu	Ld	Ku	Kd
M size								
Apr 2000	18	17	16	9	8/0	5/0	8/0	9/0
Oct 2000	9	9	6	6	10/8	8/7	8/5	10/9
Apr 2001	10	18	11	13	10/10	5/15	10/7	11/14
Oct 2001	17	17	8	9	18/12	19/12	9/10	10/10
L size								
Apr 2000	9	13	7	0	9/0	6/0	6/0	9/0
Oct 2000	20	2	2	3	10/10	10/10	9/8	8/7
Apr 2001	10	16	10	6	10/10	9/12	8/1	2/4
Oct 2001	6	9	3	1	8/0	8/4	18/20	11/20

Kd = Körsch downstream; Ku = Körsch upstream; Lu = Lockwitbach upstream; Ld = Lockwitzbach downstream.

next reproductive cycle was distinguished: intermediate stage (SP)-most cells are in spent stage; in addition, previtellogenic oocytes of the next reproductive cycle have already occurred.

The furthest developed stage of intact (*i.e.*, nonatretic) oocytes was considered to define the developmental stage of the respective individual. Thus, each individual was classified as belonging to one of the

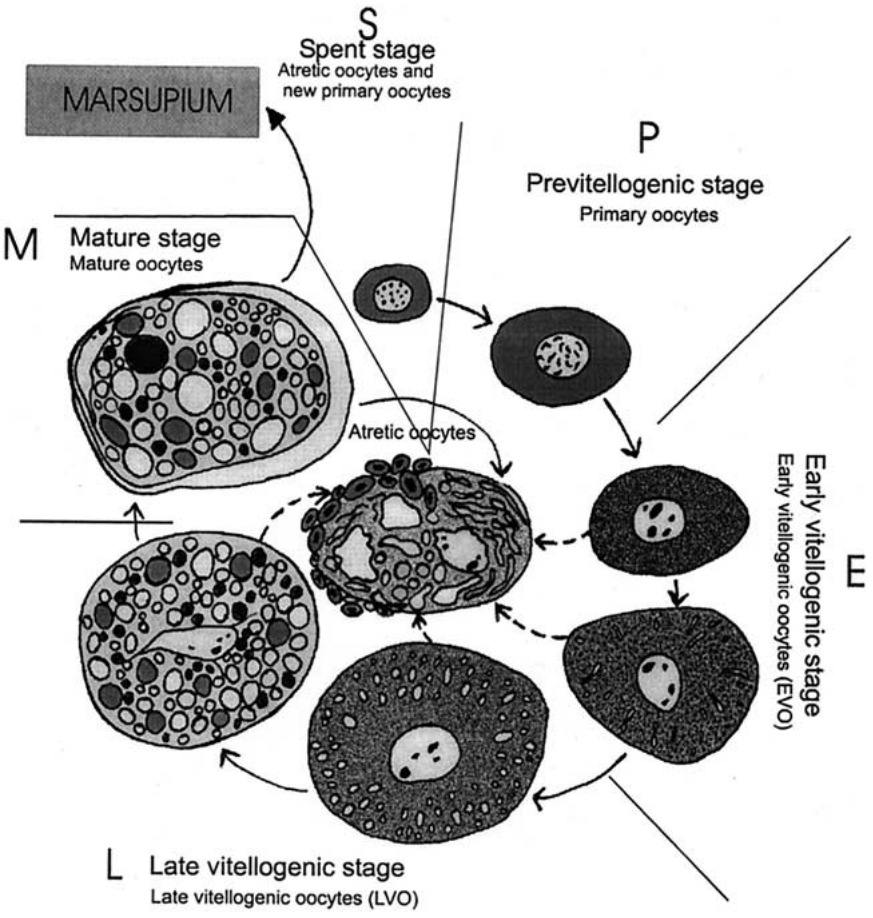

Fig. 1. Different oocyte stages according to Tan-Fermin and Pudadera (1989). Explanation is in the text.

stages previously described: SP, P, E, L, M, or S. The developmental stage of all female gammarids at a given time, location, and size class was expressed by a maturity index (mi) according to the following equation: $mi = (x_{SP} + 2x_P + 3x_E + 4x_L + 5x_M + 6x_S) * 10^{-2}$, where x_{SP}, x_P, x_E, x_L, x_M, and x_S refer to the percentages of the individuals in the respective stages SP, P, E, L, M, and S in the entire sample.

Additionally, both intact and atretic oocytes in the early vitellogenic stage and in the late vitellogenic stage were recorded. To distinguish the types of oocytes from the developmental stage of the entire individual, early vitellogenic oocytes were abbreviated EVO, and late vitellogenic oocytes were abbreviated LVO. The ratio between atretic and intact oocytes of the EVO and LVO type was calculated for each individual displaying oocytes of at least one of these two types. Furthermore, the area of intact oocytes of both the EVO as well as the LVO type in the section was determined by morphometric software (Openlab 3.0, Improvision) and a mean calculated for every individual displaying one of theses types.

Statistical Analysis

For statistical analysis we used the JMP 4.0 (SAS) software. Normally distributed data (checked by Shapiro-Wilk's test, $p \leq 0.05$) were tested for significance using Student's t test, whereas data with non-normal distribution underwent Kruskal Wallis analysis of variance (ANOVA). The α-level for significant differences was set at $p \leq 0.05$. Pairwise linear correlation analysis used the calculation of the Pearson product-moment correlations for each pair of variables ($p \leq 0.05$). Only the variables (exposure parameters) with significant linear correlation to target variables (biologic responses) were integrated in multiple regression models to explain the maximum percentage of the variability of these biologic responses. Considering the limited number of data sets and the biologic significance of extreme values, cluster analysis was performed with hierarchical clustering using Ward's minimum variance method (Milligan 1980).

Results

Differences in Stress Protein and Histology Between the Upstream and Downstream Sites

For hsp70 and hsp90, no uniform pattern was observed at the Lockwitzbach site that would give any information about the influence of the discharger. The same was true for the M-size gammarids, independent of their origin, except for a significant decrease of the hsp90 level at the Körsch downstream site in spring 2001. The L-size gammarids from Körsch, however, revealed both hsp90 and hsp70 to be tentatively or significantly decreased at Kd comparison with Ku in spring and autumn of both years (Fig. 2).

For the histologic parameters, tentative differences between the sampling sites occurred only at the Körsch site. Contrary to the heat shock proteins, the differences in histologic end points were visible only in M-size gammarids (Fig. 3). In 2000, the samplings in spring and fall revealed a higher maturity index

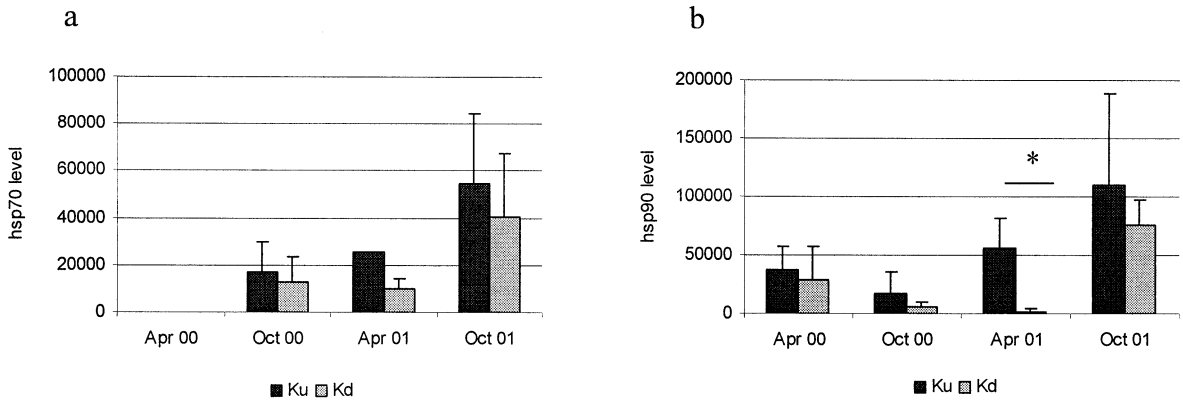

Fig. 2. (**a**) Mean hsp70 levels (= optical volume = number of pixels x average densitometric intensity of Western blot bands) and SD of L-size gammarids on Ku and Kd sites. (**b**) Mean hsp90 level (= optical volume) and SD of identical individuals. Kd = Körsch downstream; Ku = Körsch upstream.

(indicating the animals to be further along in their development) in M-size gammarids at Kd compared with Ku. In 2001, this trend hardly was visible (Fig. 4).

Because the investigated parameters maturity, oocyte area, and atresia are not entirely independent from one another, the area and atresia of LVOs largely supported the data found in the maturity analysis. The areas of LVO in particular were on average larger downstream that upstream from the sewage plant effluent at the Körsch river site (significant at $p \leq 0.05$ for April 2001) in M-size animals, whereas EVOs did not show any differences (Figs. 5 and 6). Furthermore, the level of LVO atresia in three of four samplings was higher at Kd compared with Ku, although the differences were not significant. In general, atresia at Kd was significantly higher than at Ld (Fig. 7).

Concomitantly, correlation analysis showed a positive correlation of maturity index and LVO cell area ($p = 0.002$), maturity index and LVO atresia ($p = 0.002$), and LVO cell area and LVO atresia ($p = 0.004$) in M-size gammarids. Stress protein hsp70 and hsp90 levels were found to positively correlate in L-size gammarids ($p = 0.026$) and, tentatively, M-size specimens ($p = 0.055$). The only significance in a comparison of stress proteins and histologic parameters was found in a negative correlation of hsp70 and LVO cell area in M-size gammarids ($p = 0.015$).

Cluster Analysis of Own and Literature Data

Using the data set obtained for hsp90, maturity index, EVO and LVO cell area, and EVO and LVO atresia for both M- and L-size classes, cluster analysis revealed four main clusters of respectively similar parameter patterns (hsp70 could not be included because the data set for 2000 was incomplete). These four clusters were particularly characterized by the sampling events in spring 2000, fall 2000, spring 2001, and fall 2001 (Fig. 8). In three of the four main clusters, the analysis additionally separated the two investigated streams from one another. A similar pattern of variables in locations either upstream or downstream from a sewage treatment plant, respectively, could not be found. The separation of this dataset

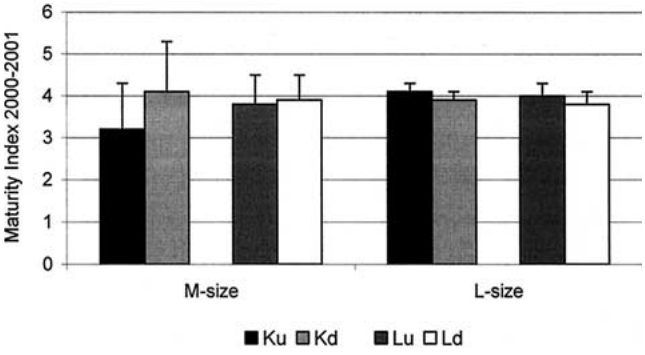

Fig. 3. Maturity index of M- and L-size gammarids at the four sampling sites: Ku, Kd, Lu, and Ld (means for for samplings during 2000 and 2001 each, and SD). Kd = Körsch downstream; Ku = - Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

into two subsets, according to the site classes M or L, resulted in different patterns in these two size classes. The subset for L-size animals did not show any clustering according to the variables sampling time, stream identity, or upstream or downstream location. In contrast, the data subset obtained for the M-size class revealed predominantly the importance of the parameter, season, which characterized the two main cluster in this analysis (Fig. 9).

The exposure situation at the four locations had been characterized regarding limnochemical data (Jungmann *et al.* 2004a) and potential endocrine disruptors (Jungmann *et al.* 2004b). Using the data obtained for the limnochemical parameters listed in Table 1 at the times of gammarid sampling, cluster analysis separated three main clusters: one fall cluster (comprising data sets for autumn 2000 and 2001) and two separated spring clusters, one for spring 2000 and the other for spring 2001. Thus, the pattern of the entire set of limnochemical parameters was season rather than site specific. The same was true for the total set of potential endocrine disruptors (Jungmann *et al.* 2004b) present in the streams at the times of gammarid sampling. The main clusters separated between the time of sampling while spatial aspects were of minor importance. At most, clusters separated between the two

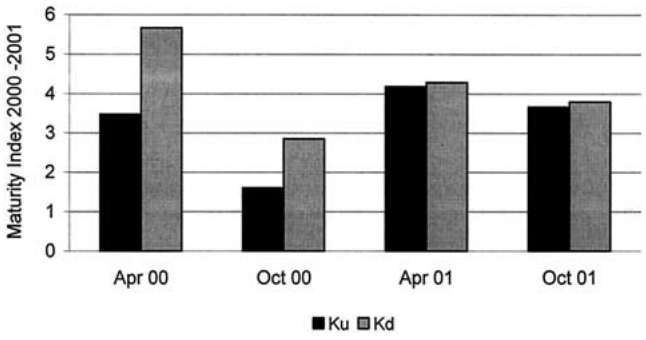

Fig. 4. Maturity index of M-size gammarids at the two sampling sites, Ku and Kd, in 2000 and 2001. Kd = Körsch downstream; Ku = Körsch upstream.

streams but not between the location of the sites up- or downstream the sewage treatment plants.

Correlation Analysis and Multiple Regression Modelling

To test not only for patterns of multiple variables but to detect exposure parameters of potential biologic relevance, correlation analysis was performed for a number of biologic parameters that were found to differ between upstream and downstream sites and the parameters of exposure (Table 1; Jungmann *et al*. 2004b). The maturation index of M-size gammarids was positively correlated with oxygen concentration ($p = 0.016$), oxygen saturation ($p = 0.010$), total hardness ($p = 0.043$), concentration of DDT ($p = 0.049$, $n = 4$), and particularly with concentration of di-n-octyl phthalate (DnOP) ($p = 0.0002$). A multiple regression model incorporating only DnOP and DDT explained 93.5% of total maturation index (M-size) variability with a significant ($p = 0.016$) contribution of DnOP to the model. Although the parameter LVO area (M size) did not show any correlation with single exposure parameters, the level of atresia in LVO (M size) was correlated positively with DnOP ($p = 0.020$) and DDT ($p = 0.004$, $n = 4$) and tentatively positively correlated with the concentration of dimethyl phthalate ($p = 0.055$). A multiple regression model using DnOP and DDT explained 78.6 % of LVO atresia (M-size) variability.

Corroborating the observed decrease of the hsp90 level downstream from the sewage plant discharge at Körsch river, hsp90 showed mainly negative correlation with exposure parameters. In M-size gammarids, the level of this stress protein did not correlate with any limnochemical parameter, but it did correlate exclusively and negatively with DnOP ($p = 0.032$) and DDT ($p = 0.026$, $n = 4$) and positively with the concentration of dibutyl phthalate ($p = 0.018$). Of hsp90 (M-size) variability, 63.2% could be explained by a multiple regression model using DnOP and DDT. In L-size animals, the only correlation of the hsp90 level was a negative relationship with the concentration of DDD ($p = 0.011$, $n = 4$, explaining 69.0 % of hsp90 [L-size] variability).

Discussion

Although different size classes of *G. fossarum* individuals were investigated separately, a high variability in the biologic

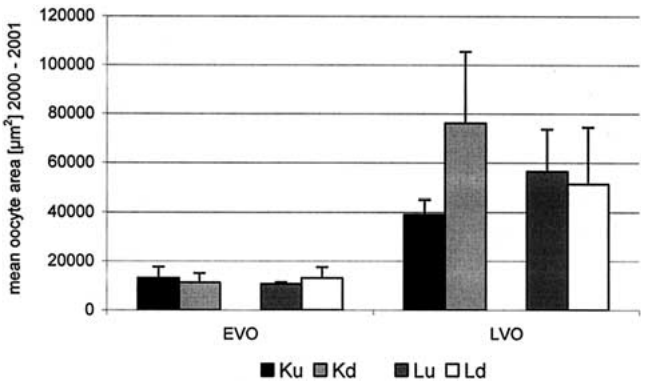

Fig. 5. Oocyte area for M-size gammarids. Means (of the four samplings in 2000 and 2001) and SDs at the four sampling sites: Ku, Kd, Lu, and Ld. Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

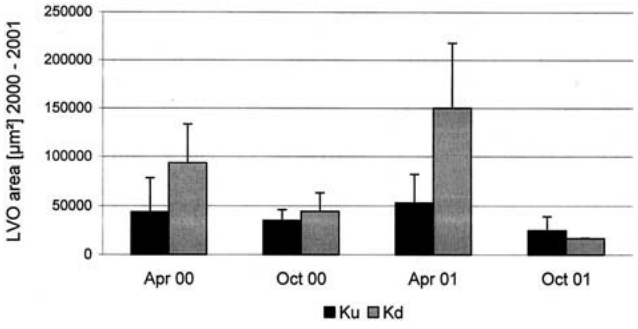

Fig. 6. Area of LVO in M-size gammarids at the two sampling sites, Ku and Kd, in 2000 and 2001. Means and SDs. Kd = Körsch downstream; Ku = Körsch upstream; LVO = late vitellogenic oocytes.

parameters was recorded, which prevented significant differences in most cases. This variability results at least partially from differences in the developmental status of individuals within a distinct size class (Pöckl and Humpesch 1990). Nevertheless, effects of the respective exposure situations on gonad development and hsp90 level, both parameters related to the endocrine system, could be found in the Körsch river: differences in maturity of oocytes, larger late vitellogenic oocytes, increasing atresia, and decreasing hsp90 levels occurring downstream from the discharge of a sewage treatment plant. At the Lockwitzbach site, these effects were not found. Indeed, the main difference between the two rivers was in the amount of discharged water, which is much higher at the Körsch than the Lockwitzbach site. As a consequence, a higher estrogenic potential introduced by the sewage plant effluents was measured at the Körsch site (Jungmann *et al*. 2004b). Even though an influence of sewage plant effluent on the investigated biologic parameters is likely at least for the situation at the Körsch site, solely for this reason the effects must not necessarily be related to analyzed xenohormones because a number of other pollutants, e.g., PAHs, PCBs, and pesticides, are introduced into this river as well (Honnen *et al*. 2001). Nevertheless, there is evidence for the interaction of external parameters with the internal reproductive cycle in *G. fossarum*.

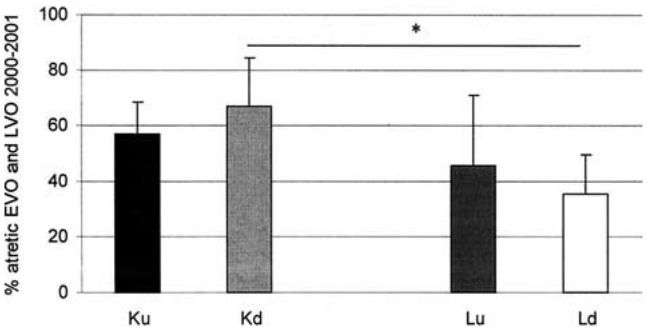

Fig. 7. Percentage of atretic oocytes (means of atresia in both LVO and EVO for all four samplings during 2000 and 2001 and SDs) in M-size gammarids at the four sampling sites: Ku, Kd, Lu, and Ld. EVO = early vitellogenic oocytes; Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

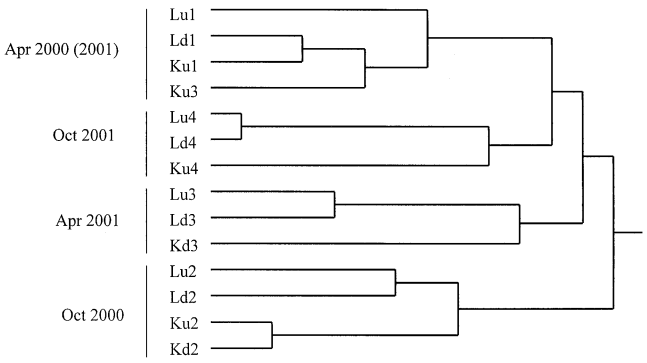

Fig. 8. Dendrogram according to the cluster analysis of the data set for maturity index, area LVO, area EVO, atresia LVO, atresia EVO, and hsp90 in both M- and L-size gammarids. 1 = April 2000; 2 = October 2000; 3 = April 2001; 4 = October 2001. Mean clusters are indicated by vertical lines. EVO = early vitellogenic oocytes; Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream; LVO = late vitellogenic oocytes.

At the cellular and individual level, biologically linked parameters such as maturity stage, the size of more developed oocytes and the number of atretic LVO cells (symbolizing leftover oocytes that have not been released into the brood pouch) correlated in a positive way. In contrast to noncontaminated laboratory experiments (Schirling *et al.* 2004), however, the field situation resulted in a disruption of the endogenous hsp90 cycle (initially a positive relationship with increasing maturity) and the female reproductive cycle (negative correlation instead). Furthermore, in the investigated field sites, hsp90 and hsp70 levels were found to correlate rather in a positive way (in contrast to the negative correlation under controlled laboratory conditions), presumably representing hsp90 as acting as a stress protein along with hsp70. Although it is known that heavy pathologic impact usually affects biochemical processes, the uncoupling of oocyte maturation and the hsp90 cycle in this case cannot be interpreted as a result of histopathology. Histology did not reveal pathologic changes in the gonads of *G. fossarum*. Furthermore, in this study the abundance of *G. fossarum* was found to be highest at the Kd sampling site (Ladewig and Nagel 2004), possibly because of rich nutritional supply. If the decrease of hsp70 and hsp90 had been related to an irreversible cessation of protein synthesis, such an injured population would hardly be stable. The increase of atresia in LVO cells also must be interpreted as a result of resorption of oocytes that have not been released into the marsupium (because of the spatial limitations of this brood pouch) rather than as resulting from histopathologic impact (as described by Yashodhara 2002). Otherwise, EVO cells or other earlier oocyte stages would have exhibited increased atresia as well. Therefore, we assume that other potential endocrine disruptors (which have not been analyzed) and abiotic parameters interact with regulation processes in a nondestructive way, thus resulting in a high temporal and spatial variability of parameters related to the reproductive cycle.

Indeed, as shown by cluster analysis, the pattern of exposure parameters, represented by the limnochemical and analytic chemistry data, varied predominantly temporally. Therefore, the influence of the wastewater discharge was only visible for single parameters but not for the entire pattern (thus "disguising" the possible importance of single compounds to some extent). Under these circumstances, it is reasonable that the general pattern of biologic responses depended predominantly on temporal aspects such as the season or the sampling time. Although the selection of investigated biologic parameters took place particularly to trace "endocrine" effects, a number of these parameters consequently must be regarded as to have been considerably influenced by those environmental factors that do not specifically target the endocrine system. However, correlations of single parameters in specific size classes—such as the stage of maturity, the size of LVO cells and the ratio of LVO atresia in animals of 6–9 mm in length, as well as the hsp90 level in animals >9 mm with single-exposure parameters—indicate the importance of potential xenohormones for *G. fossarum* development. A number of observations support this interpretation: As shown by Watts *et al.* (2002), the genus *Gammarus* spp. is *per se* susceptible to estrogenic compounds. Responses of the mentioned cytologic and biochemical parameters were only observed at the Kd sampling site, characterized by an increased estrogenic potential. In a parallel study, Ladewig and Nagel (2004) showed demographic parameters in *G. fossarum* to be altered at the Kd sampling site. We found a significant correlation of different mentioned cellular and molecular markers always with the same compounds (DnOP and DDT or its metabolite, DDD), and models showed a high potential of these exposure variables to explain the biologic responses. Correlation with other abiotic variables, such as temperature, pH, phosphorous and nitrogen compounds, etc., was lacking, thus indicating the minor importance of these parameters for the mentioned biotic responses. In a laboratory experiment with a single potential estrogenic compound, bisphenol A, the identical tendency of *G. fossarum* to respond with a higher maturation index, larger LVO cells, a higher rate of LVO atresia, and a decreasing hsp90 level (Köhler and Triebskorn 2004; Schirling and Köhler 2004) mirrored the situation at the downstream location at the Körsch site.

Because mechanistic studies on the interaction of potential xenohormones with the endocrine system in *Gammarus* spp.

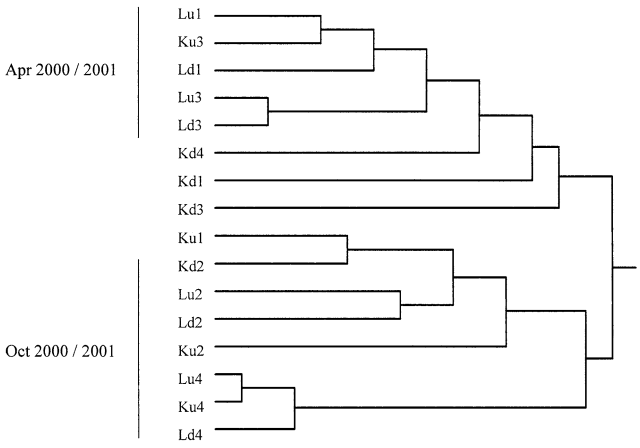

Fig. 9. Dendrogram according to the cluster analysis of the dataset for maturity index, area LVO, atresia LVO, and hsp90 in M-size gammarids. 1 = April 2000; 2 = October 2000; 3 = April 2001; 4 = October 2001. Mean clusters are indicated by vertical lines. Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream; LVO = late vitellogenic oocytes.

are still lacking, these observations give presumptive evidence only. The fact that one size class of gammarids showed an effect on one criterion and a different size class responded with another may well reflect the complexity of the pollution situations examined and the potential for interactions at the different sampling conditions. Clearly, more single-pollutant laboratory studies are needed in which specific correlation with response criteria can be made. However, in view of the precautionary principle, the mentioned single biologic parameters that were found to be influenced in this study can be recommended as indicators of possible influence of potential xenohormones on *G. fossarum*. Considering the technical aspects, this certainly is advantageous for biotests aiming at the detection of potential endocrine effects on crustaceans. As shown in this study, the field situation, however, is much more complex, and effects on single cellular or biochemical parameters may be hidden or even compensate for seasonal cycles of gonad development

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