Biased sex ratio in the European eel (Anguilla anguilla) swimbladder parasite Anguillicola crassus, experimentally induced by 11-ketosterone

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RH: RESEARCH NOTE

BIASED SEX RATIO IN THE EUROPEAN EEL (*ANGUILLA ANGUILLA*) SWIMBLADDER PARASITE *ANGUILLICOLA CRASSUS*, EXPERIMENTALLY INDUCED BY 11-KETOTESTOSTERONE

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ABSTRACT: Parasites are intimately connected to the host in which they live, and some may be affected by the polluted environment of their host. The present study describes the effect of a steroid hormone (11-ketotestosterone) on the sex ratio of the invasive hematophagous nematode *Anguillicola crassus* Kuwahara, Niimi & Itagaki, 1974, when experimentally injected to European eels, *Anguilla anguilla*, Our results showed that this steroid induced a significant male-biased ratio in the nematode *A. crassus* infrapopulations, suggesting that the presence of endocrine disruptors in the environment may lead to skewed sex ratios among parasites.

The biological effects of environmental pollutants on aquatic organisms have recently become a major concern. Pollutants are being discharged continuously into water throughout the world, resulting in substantive changes in aquatic habitats, mainly in freshwater and coastal areas such as estuaries and lagoons. The toxicity of these chemical and biological substances has mostly been evaluated (both in laboratory and field conditions) in fishes, revealing disturbances in feeding (Eddy, 2005), growth (Robinet and Feunteun, 2002), immunity (Dunier and Siwicki, 1993), and reproduction (Jones and Reynolds, 1997; Sumpter, 1997). A great interest was taken in the effects of toxic pollution

on parasites, for 2 major reasons. First, parasites, in particular metazoans, may be reliable biological indicators of water contamination (Poulin, 1992; MacKenzie et al., 1995; Marcogliese 2005). Second, the impacts of parasites on their hosts can be positively or negatively affected by pollution (Sures, 2006). However, the former considerations demonstrated the need to understand how pollutants might directly or indirectly influence the prevalence, intensity, and pathogenicity of a parasite. General (non-exclusive) trends have emerged from different studies. Thus, on the one hand, pollutants may increase parasitism by impairing the host immune response or by favoring the survival and reproduction of the intermediate host (Khan and Thulin, 1991; Poulin, 1992; Sures, 2006). On the other hand, pollutants may decrease parasitism by being more toxic to parasites than to their (final) hosts (for example high toxicity to free-living stages), negatively affecting intermediate hosts, or altering the host physiology (Khan and Thulin, 1991; Poulin, 1992; Sures, 2006). In any case, studies are required for each new pollutant/parasite/host system.

The present study focused on steroid hormones, which are considered as emerging pollutants (Lopez de Alda et al., 2003). A recent investigation conducted in North America, steroid compounds such as 17α and β-estradiol, estriol, estrone, progesterone, and testosterone, which are generally considered as important reproductive hormones in vertebrate animals, were the ones most frequently found as pollutants in aquatic habitats, and occurred in the highest concentrations (Kolpin et al., 2002). These hormones should disrupt important endocrine-mediated processes and would thus have an effect on various reproductive functions, potentially leading to masculinization or feminization of organisms, as well as biased sex ratios in populations (Christiansen et al., 2002; Khanal et al., 2006; Martinovi et al., 2007).

Specifically, 11-ketotestosterone (11-KT) belongs to the family of 11-oxygenated androgens that are typically produced in fish testes (Borg, 1994). It is known that aquatic environments are exposed to 11-oxygenated androgen contamination because of an increase of intensive aquaculture practices (Foresti, 2000; Boyd et al., 2005). Moreover, several xenobiotics that may disrupt endocrine functions are extensively discharged in the environment due to agricultural and industrial practices (Kime, 1999; Zala and Penn, 2004). We, therefore, attempted to assess the biological effects of 11-KT on the invasive parasite species, *Anguillicola crassus* Kuwahara, Niimi & Itagaki, 1974, a parasite of the swim bladder of European eels (*Anguilla anguilla* Linnaeus, 1758). In addition to being an invasive species, *A. crassus* is an hematophagus species, bringing it into direct contact with the host blood, in which potential pollutants that may be circulating. Because the parasite is also gonochoric, the aim of the study was to investigate a potential effect of the 11-KT on the parasites' sex ratio.

Eels were caught in July 2005 at the Palavasian Lagoons (43.54°N 3.92°E, Hérault, France) by a professional fisherman. Epidemiological surveys carried out in July 2004 on 20 eels and 119 more in June and July 2005 revealed surprisingly low prevalences of *A. crassus*, and no more than 2 parasites per host (unpubl. obs.). During the July 2005 collection, the eels were sorted in order to select only males. Catch effort was focused on eels about to silver (to metamorphose before their oceanic migration to the Sargasso Sea) in order to limit individual variability. The selection was based on 4 criteria, i.e., the total body length (L_T), the ocular index (I_O), the skin coloration, and the differentiation of the lateral line. Eels that were approximately 40 cm and that exhibited ocular hypertrophy, a differentiated lateral line, and a contrasting color (Acou et al., 2005; Durif et al., 2005), were brought to the laboratory in oxygenated lagoon water and transferred to 10, 100-L

tanks filled with artificial sea water (S = 37 g/L). They all received a mebendazole (Sigma; St. Louis, Missouri) treatment (1 mg/L for 24 hr) for monogeneans (Buchmann 1993). Freshwater copepods (*Cyclops* spp.) were collected at the Villeneuve-de-la-Raho Lake (42.63° N 2.90° E, Pyrénées-Orientales, France) with a plankton net. They were fed with second stage (L2) larvae of *A. crassus* recovered from swim bladders of naturally infected eels (around 10 L2 larvae per copepod). They were then maintained at 24 C in oxygenated water and fed once a day with *Paramecium* sp. Third stage larvae developed by 11 days post-infection. The L3 stage was confirmed microscopically by the presence of a brace-shaped sclerified structure at the anterior end of the larvae, called the "buccal ornamentation" (Blanc et al., 1992). At this point, L3 larvae were harvested from the copepods with a pestle in physiological serum (8.5‰) and counted using a binocular microscope.

Experimental infections of the eels were performed after at least 1 wk of acclimatization in aquaria. The eels were anesthetized (0.1 ml/L of Eugenol) and measured (total length, L_T). The horizontal (D_h) and vertical (D_v) eye diameters were measured to the nearest 0.1 mm on the left side of exposed eels. The ocular index (I_O) (Pankhurst 1982) was calculated as: $I_O = [((D_h+D_v)/4)^2*\pi/L_T]*100$. Batches of 50 L3 larvae were prepared in physiological serum. The larval suspension was drawn into a syringe with a blunt cannula and orally administered intubated into the eels' stomachs. The eels were infected with 50 L3 (1 syringe of 50 L3). Of the 36 exposed eels, 16 received an injection of 11-ketotestosterone (11-KT, Sigma); 2 µg of 11-KT per 1 g of eel, homogenized in about 0.5 ml of physiological serum (6‰), were injected each wk into the body cavity (S. Dufour, pers. comm.). This treatment began 1 wk after the experimental infections and continued for 5 wk. Following the same timing protocol, the remaining 20 eels were injected just with

serum.

At time of initial exposure to the third-stage larvae, the biological characteristics of the eels were $352< L_T <438$ mm and $6.0< I_O <8.7$ for (further) non-11-KT treated eels (N=20) and $343< L_T <449$ mm and $5.5< I_O <8.9$ for (further) 11-KT treated eels (N=16). These differences in treated and untreated eels were not significantly different (Mann-Whitney *U*-tests, L_T: U=130.5, P>0.05 and I_O: U=160.0, P>0.05). After 6 injections, the eels were anesthetized (0.1 ml / l of Eugenol), weighed and measured (total length) to the nearest 0.1 g and mm, respectively, then instantly killed by beheading.

Five mo post-infection, swim bladders were removed and examined using a binocular microscope to recover parasites. The developmental stages, i.e., L3 larvae, L4 larvae, and/or adults, as well as sex of the adults, were determined. The recovery success was calculated as the number of recovered parasites divided by the number of intubated L3 larvae. The male-ratio was calculated as the number of recovered males divided by the number of recovered adults, for each eel. Both male and female infrapopulations were singly weighed to the nearest mg. We calculated a mean individual male (female) biomass per infected eel, considering all the males (females), divided by the number of males (females) recovered in each fish. The mean individual male and female biomass and infrapopulation biomass ± Standard Deviation for each sex were further calculated for 11-KT treated and untreated eels. Non-parametric Mann-Whitney *U*-tests were performed comparing 11-KT treated and 11-KT untreated eels for observed male-ratios, recoveries of male/female parasites, and male/female biomass data.

When the 2 sexes are combined, the mean number of parasites recovered was

7.4±5.2 (min-max =1-19). The mean recovery success was 0.13±0.09 for 11-KT untreated eels (Ø) and 0.19±0.12 for 11-KT treated (11-KT) eels, and are not significantly different ($n_{Ø}$=20, $n_{11\text{-}KT}$=16, *U*=116, *P*>0.05). The mean number of males was 2.7±2.2 (min-max=0-8) in untreated eels and 5.4±3.9 (min-max=0-14) in 11-KT injected eels (Fig. 1). The mean number of females was 3.4±2.3 (min-max=0-7) in untreated eels and 3.7±3.2 (min-max=0-12) in 11-KT injected eels (Fig. 1). Mann-Whitney *U*-tests revealed that the mean number of recovered males was significantly higher in 11-KT eels than in Ø eels ($n_{Ø}$=20, $n_{11\text{-}KT}$=16, *U*=95, *P*=0.037). However, the numbers of recovered females in Ø and 11-KT eels were not different ($n_{Ø}$=20, $n_{11\text{-}KT}$=16, *U*=156, *P*>0.05). The mean male-ratios were 0.42±0.27 and 0.59±0.27 for Ø and 11-KT eels, respectively. A Chi square test did not indicate a significantly biased sex ratio in untreated eels (χ^2=0.6, ddl=1, *P*=0.44), while it was significantly male biased in 11-KT treated eels (χ^2=34.1, ddl=1, *P*<0.001). Figure 2 shows the male and female *A. crassus* individual biomass in Ø and 11-KT eels. Mann-Whitney *U*-tests did not revealed any significant differences ($n_{Ø}$=17 and 18, and $n_{11\text{-}KT}$= 15 and 14, for either male and female mean biomass, respectively, *P*>0.05) between Ø and 11-KT eels.

Our results revealed a significant male biased sex ratio in the nematode *A. crassus* infrapopulation when eels received a 11-ketotestosterone treatment. Studies on the sex ratio of the worm in the field are rare, but the results suggest a naturally equilibrated sex ratio (Belpaire et al., 1989; data not shown). Moreover, we did not observe a significantly biased sex ratio in untreated eels. The distortion observed in injected eels is due to the development of a larger number of males since the overall

number of females did not decrease in 11-KT treated eels. Moreover, the bias of the male-ratio was not linked to any change in collective mean male and female biomass. This suggests that the injection of 11-KT into the hosts induced a distortion in the blood sucking parasite sex ratio by promoting male development.

We suggest 2 (non-exclusive) hypotheses to explain the results obtained for the host parasite model employed in the present study, and the experimental protocol followed. First, the 11-KT treatment may have increased the eel susceptibility to the nematode by affecting the immune system. Second, the 11-KT treatment may have favored the development of male parasites.

The deleterious effect of dihydrotestosterone and testosterone on both innate and acquired immunity has been well studied in mammals, i.e., sex hormone receptors are known to be localized in immune cells such as lymphocytes, macrophages, granulocytes, and mast cells (see Klein, 2004, for a review). However, very little is known with respect to teleost fishes. However, recent studies failed to show an effect of androgen treatments on some components of the immune system in tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) (Law et al., 2001) and in tench (*Tinca tinca*) (Vainikka et al., 2005). Furthermore, an immune effect should rather have had consequences for infectivity of the nematode and thus have led to an increase in both the male and female components of the parasite infrapopulation. However, the number of females recovered was not different between treated and untreated eels. Nonetheless, we cannot reject the immunity hypothesis, since other pollutants have been shown to act as immunomodulators in the eel. For example, Sures and Knopf (2004) demonstrated that European eels experimentally infected with L3 larvae of *A. crassus* were not able to produce specific antibodies when they were

simultaneously exposed to polychlorinated biphenyls (PCBs).

The second hypothesis that could explain the male biased sex ratio would be an enhancement of the development of male parasites, by modifying the density-dependent relationships between parasites at the infrapopulation level. Two density-dependent mechanisms are known to constrain the infrapopulation size of *A. crassus* within the eel (Ashworth and Kennedy, 1999). First, adults in the swim bladder of eels were shown to inhibit the development of L3 larvae, in cases of heavy infections, i.e. more than 20 parasites. Second, density-dependence limits the number of female worms reaching maturity because of large size of already present adult females. This latter "crowding effect", was found by these authors at a mean number of adults ranging between 3 and 9 and could occur in our studied infrapopulations where the mean number of adults ± standard deviation (min-max) was 7.4 ± 5.2 (1-19). This hypothesis is supported by the blood diet of the nematode, which should account for the transfer of 11-KT from the digestive system to the nematode, as well as the presence of a large number of steroid receptors among various species of nematodes (Höss and Weltje, 2007).

Our results suggest an enhancement in the development of male parasites, but we do not actually know on which developmental stage the 11-KT has an effect. Following the findings of Ashworth and Kennedy (1999) on the development of L3, the 11-KT may have had an effect on the relationship between L3 larvae and adults. Moreover, their findings on the constraint of the number of gravid females, suggest the existence of a competition between the adult stages. Both intraspecific interactions (larvae vs. adults or adults vs. adults) involve independent mechanisms that could occur simultaneously. However, more experimental work, especially a molecular protocol to

determine the sex of L3, could help in answering these questions.

The fact that 11-KT had no effect on the mean parasite biomass suggests that this hormone does not affect morphological characteristics of the nematode. More work is needed to test if other life history traits (for males in particular), i.e., acceleration of growth and acquisition of puberty, or an increase of gonad size and fecundity, would be affected by 11-KT.

In conclusion, these preliminary results should be confirmed in field studies by comparing the parasites' sex ratios between polluted and unpolluted habitats.

The authors would like to thank Mr Molle for supplying with eels. This work was financially supported by the French Ministère de l'écologie et du développement durable, the CNRS and the University of Perpignan Via Domitia. G.F. was financially supported by a grant from the French Ministère de l'enseignement supérieur et de la recherche.

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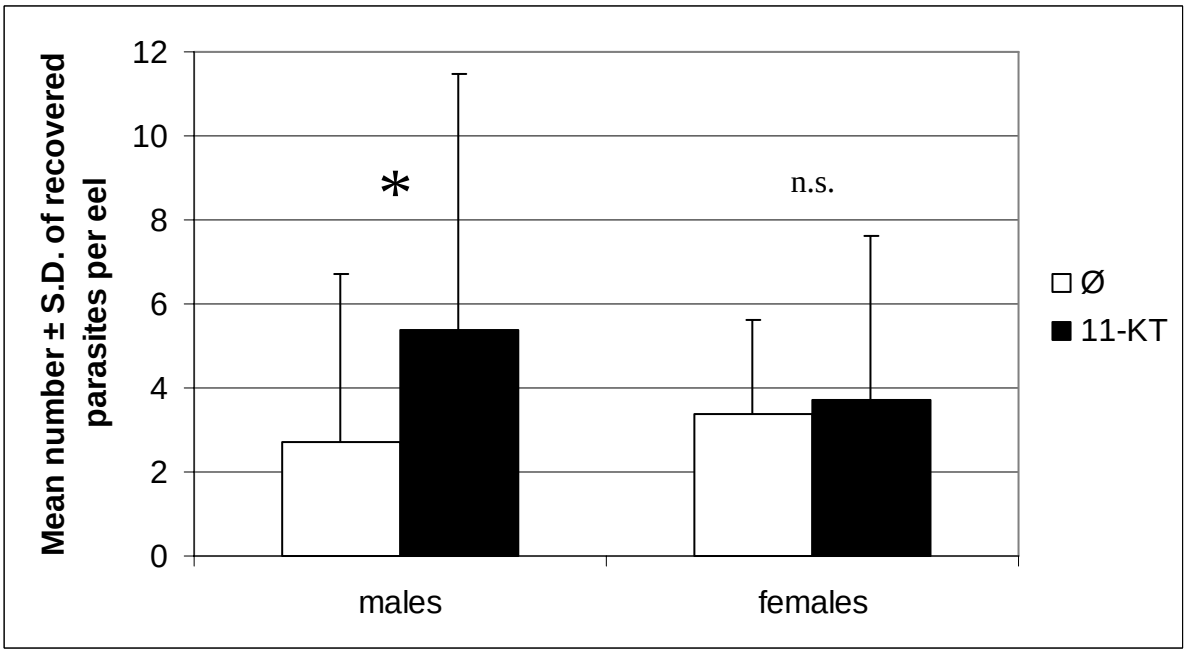

Fig. 1. Mean number (± SD) of male and female *A. crassus* recovered in experimentally infected eels, after a 6 weeks-long 11-ketotestosterone treatment (black bars, N = 16 eels) or no treatment (white bars, N = 20 eels). "*"$p<0.05$ (Mann-Whitney test between treated and non-treated eels). S.D., standard deviation; n.s., not significant.

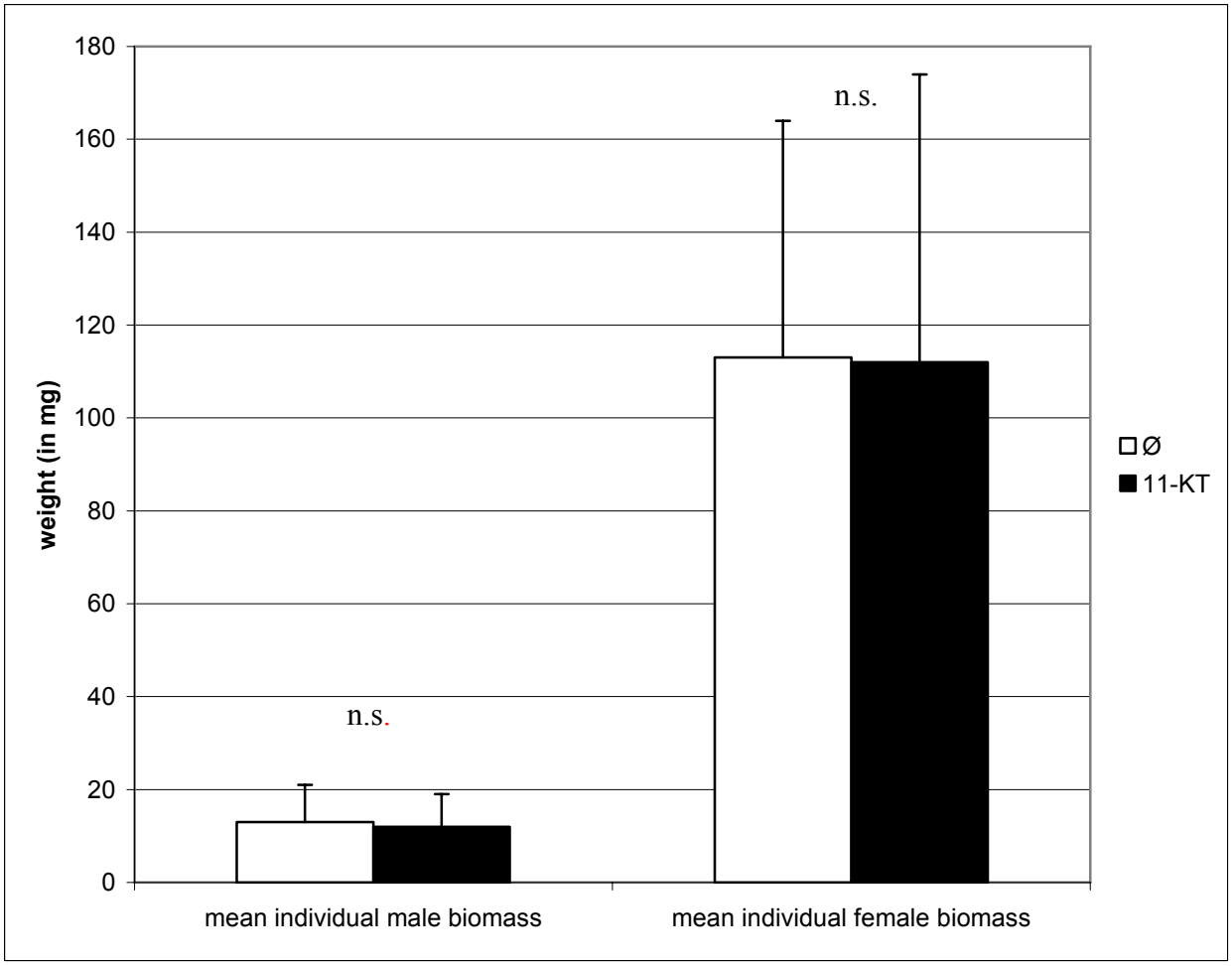

Fig. 2. Male and female *A. crassus* individual biomasses in experimentally infected eels, after a 6 weeks-long 11-ketotestosterone treatment (black bars, N = 15 and 14 eels for male and female data, respectively) or no treatment (white bars, N = 17 and 18 eels for male and female data, respectively). No significant difference (n.s.) was found between treated and non-treated eels (Mann-Whitney test).