生 态 学 报 2010,30(20):5537—5543
Acta Ecologica Sinica

壬基酚暴露对斑马鱼求偶行为与繁殖成功率的影响

夏继刚,牛翠娟*
(生物多样性与生态工程教育部重点实验室,北京师范大学生命科学学院,北京 100875)

摘要:壬基酚(NP)是普遍存在于水生生态系统中的一种环境内分泌干扰物。研究了不同浓度下(0,0.1,1,10,50,100 μg/L)NP暴露对斑马鱼(*Danio rerio*)求偶行为与繁殖成功率的影响。结果表明,NP暴露对斑马鱼求偶行为与繁殖成功率影响显著,100 μg/L NP暴露显著减少斑马鱼求偶时间、降低产卵量与受精率($P < 0.05$)。斑马鱼产卵量与求偶总时间、平均每次求偶时间、长于5 s的求偶时间显著正相关($P < 0.05$),与求偶频率不相关($P = 0.951$)。NP暴露可能通过影响斑马鱼求偶时间,进而影响产卵量。斑马鱼求偶时间有望作为评估水体NP污染有效的生物标记。

关键词:壬基酚;求偶行为;繁殖成功率;斑马鱼

Effects of exposure to nonylphenol on courtship behavior and reproductive success of zebrafish (*Danio rerio*)

XIA Jigang, NIU Cuijuan*
Ministry of Education Key Laboratory of Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, *China*

Abstract: Endocrine-disrupting chemicals (EDCs) pose a serious risk to the health of wildlife. Nonylphenol (NP), one of the most conerned EDCs, is a degradation product of nonylphenol ethoxylates (NPEs), which has been extensively used in industrial and household production and is commonly released from industrial and municipal sewage treatment plants into the aquatic environment. In recent years, studies demonstrated that NP is capable of mimicking the biochemical behavior of natural estrogen and interacting with the estrogen receptor.

Behavior repertoire is the cumulative manifestation of biochemical, physiological and environmental cues, reflecting the outcome of an animal's integrated physiological response and biochemical alterations resulted from contamination. Inappropriate behavioral performance caused by EDCs is of high ecological significance, as toxicants can often impair or even eliminate the behavioral patterns that are necessary for the fitness and survival of the whole population. Courtship behavior in fish is one of the key endpoints for assessing the effect of EDCs. If courtship behavior is suppressed by EDCs, spawning potential would be depressed and, subsequently, population structure and dynamics also might be disturbed. Thus, it is important to evaluate the effects of EDCs on courtship behavior of fishes. However, a standard protocol for assessing the effect of EDCs on courtship behavior in zebrafish (*Danio rerio*) has not yet been established.

The present work examined the effects of NP on courtship behavior and reproductive success of zebrafish. Fish were randomly divided into six groups. Five groups were receiving 0.1, 1, 10, 50 and 100 μg/L nominal concentrations of NP for 40 d, respectively. A sixth control group was given the same treatment as the other five groups, but received acetone only. The nominal concentration of acetone in waterborne didn't exceed 10 μL/L. The contaminants were administered via the water, as 50% of the water was daily exchanged and replaced with water contaminated with NP. Courtship behavior was observed during a 15 min period. Time of courting, average time of each courting, time of courting that exceeds 5 sec., frequency of courting, frequency of courting that exceeds 5 sec. and frequency of courting that less than 5 sec. were used to

基金项目:国家自然科学基金重点项目(No.40632009)
收稿日期:2009-09-21; **修订日期**:2010-01-28
* 通讯作者 Corresponding author. E-mail: cjniu@bnu.edu.cn

quantify the courtship behavior performance of zebrafish. The fecundity and fertility were studied to assess reproductive success. Significant differences in courtship behavior and reproductive success were observed in different NP exposure groups. The time of courting, fecundity and fertilization rate were significantly inhibited in 100 μg/L NP exposure group ($P < 0.05$). A positive correlation ($P < 0.05$) was found between the fecundity and the time of courting, including the total time of courting ($R = 0.637$), the average time of each courting ($R = 0.414$) and total time of courting that exceeds 5 sec ($R = 0.619$), whereas no significant correlation ($R = 0.009$, $P = 0.951$) was found between the fecundity and the frequency of courting. It was indicated that the effects of NP on fecundity of zebrafish may partly be attributed to the shortened time of courtship. Time of courtship might be promising biomarker or indicator of NP contamination in aquatic ecosystem.

Key Words: nonylphenol; courtship behavior; reproductive success; zebrafish

环境内分泌干扰物(endocrine disrupting chemicals, EDCs)严重威胁动物的生命活动以及人类的生殖健康,正日益成为人们高度关注的环境问题。壬基酚(Nonylphenol, NP)是壬基酚聚氧乙烯醚(NPEs)的降解产物,广泛应用于造纸、纺织、避孕品、洗涤剂和化妆品等的生产中,与人们的日常生活关系十分密切[1]。大量研究表明,NP 具有雌激素活性,是一种环境内分泌干扰物[2-3],NP 暴露能够抑制性腺发育[4],改变性激素水平[5],诱导雄鱼肝脏卵黄蛋白原(VTG)生成[6]等,尚未见 NP 对鱼类繁殖行为毒理学效应的文献报道。

行为是机体响应外界环境变化的终端反应,它整合了环境污染物在分子、生理、生化等水平上的效应,超越了生物组织的单一层次,从而直接反应出 EDCs 对动物适合度的影响,具有重要的生态学意义[7-8]。鱼类的繁殖行为是评估水体 EDCs 生态毒理学效应的重要终点[9],EDCs 破坏繁殖行为,将潜在的影响动物的繁殖输出,进而影响种群动态[10]。

斑马鱼(*Danio rerio*)是一种小型热带鱼,由于其世代周期短,产卵量大、易收集、易饲养等特点,如今已被作为模式生物广泛用于生物学研究和各种化学品的毒性测试,是水体污染监测的理想指示生物[11]。Larsen 等[12]最早以运动距离、运动速度等指标对斑马鱼求偶行为进行了量化,然而这种方法是不完善的,因为没有将日常行为(非性行为,例如休息、随意游动等)与求偶行为区分开来,在评估求偶行为时剔除日常行为。本文提出了新的斑马鱼求偶行为量化方法,在时间尺度上,以求偶总时间、长于 5 s 的求偶时间、平均每次求偶时间,以及求偶频率等为指标,定量评估了 NP 暴露对斑马鱼求偶行为的影响,并对求偶行为与产卵量作相关分析,旨在为 NP 对鱼类繁殖行为的生态毒理学效应提供理论依据。

1 材料与方法

1.1 实验动物

斑马鱼(AB 品系)由北京大学斑马鱼中心提供,饲养方法参照 Westerfield[13]。实验条件如下:实验用水为充分曝气脱氯并经活性炭过滤的自来水,水温(25 ± 1)℃,pH(7.6 ± 0.4),溶解氧高于 4 mg/L、总硬度 220—250 mg/L,光周期 14 L:10 D,饲养密度 $\rho < 1$ g/L,每日投喂 2 次冰冻红虫(tubifex)(北京绿环观赏鱼用品公司),投喂 15 min 后,吸去残饵。选取 120 d 以上,健康状况良好的斑马鱼进行实验,平均体重雄鱼为(0.41 ± 0.003)g、雌鱼为(0.49 ± 0.004)g。

1.2 暴露试验

暴露容器为方形玻璃缸(24 cm × 8 cm × 20 cm)。为避免产生应激反应,正式暴露试验前,试验鱼驯养 2 周,使其适应试验环境。将壬基酚(NP, Sigma 公司)以丙酮(分析纯)为助溶剂,配置成 10 mg/mL 的母液,4 ℃避光保存,待用。试验设置 6 个浓度梯度,分别为对照组(0)、0.1、1、10、50、100 μg/L。其中,对照组丙酮浓度为 10 μL/L,低于 Hutchinson 等所推荐的最大浓度[14]。每个浓度梯度组随机放入体质健康、规格一致的斑马鱼 15 尾,每个处理设 3 个平行组。采用半静态暴露装置,每天换水 50%,持续暴露 40 d。

1.3　行为测试

繁殖行为测试在 1 L 的烧杯中进行,在接近烧杯底部 1 cm 处铺有一层铜网,以防产下的卵被斑马鱼吃掉。烧杯使用前用相应浓度的暴露液浸泡。行为测试的前一晚,将各烧杯加水至 900 mL,水体的 NP 浓度与暴露期间相一致,水温(25 ± 1)℃。随机选取每一个浓度梯度下的斑马鱼雌雄各 8 尾,分别按雌雄比 1:1 配对放入 8 个烧杯中过夜(每个烧杯中含有斑马鱼雌鱼、雄鱼各 1 尾)。由于斑马鱼是光周期产卵,求偶行为及产卵主要发生在光照开启后的 30 min 内[15]。本实验于次日光照开启 1 min 后开始用摄像机(SONY, JAPAN)拍摄记录斑马鱼的求偶行为,连续拍摄 15 min,录像资料随后在电脑上进行分析,通过秒表软件分析记录各行为指标的时间及频次。本文以 15 min 内的求偶总时间、求偶次数、平均每次求偶时间、长于 5 s 的求偶次数、长于 5 s 的求偶时间、短于 5 s 的求偶次数等为指标来定量求偶行为。

1.4　繁殖率

求偶行为发生 4 h 后移出亲鱼,收集受精卵并统计产卵量与受精率,用以评估繁殖率。

1.5　数据处理

应用软件 SPSS for Windows 16.0(SPSS Inc., USA)进行统计分析。首先对数据进行正态性和方差齐性检验,用单因素方差分析(ANOVA)和最小显著差数法(LSD)检验差异显著性;如果不符合正态分布或方差不齐,则采用 Kruskal-Wallis 检验。采用偏相关分析检验各求偶行为指标与产卵量的关系。各组数据均用平均值 ± 标准误表示,显著性水平设置为 $\alpha = 0.05$。

2　结果

2.1　求偶行为

试验结果显示:NP 对斑马鱼求偶时间有显著影响($P < 0.05$)(图 1)。暴露于高浓度 NP(100 μg/L)的斑马鱼求偶时间显著减少($P < 0.05$),低于 100 μg/L 的 NP 暴露对斑马鱼求偶时间无显著影响($P > 0.05$)(图 1A)。NP 暴露对斑马鱼长于 5 s 的求偶时间影响显著($P < 0.05$),并呈“∩”型毒理曲线;高浓度(100 μg/L)NP 暴露导致斑马鱼长于 5 s 的求偶时间显著减少($P < 0.05$),而中等浓度(10 μg/L)NP 暴露导致斑马鱼长于 5 s 的求偶时间显著增加($P < 0.05$)(图 1B)。不同浓度 NP 暴露对斑马鱼平均每次求偶时间的影

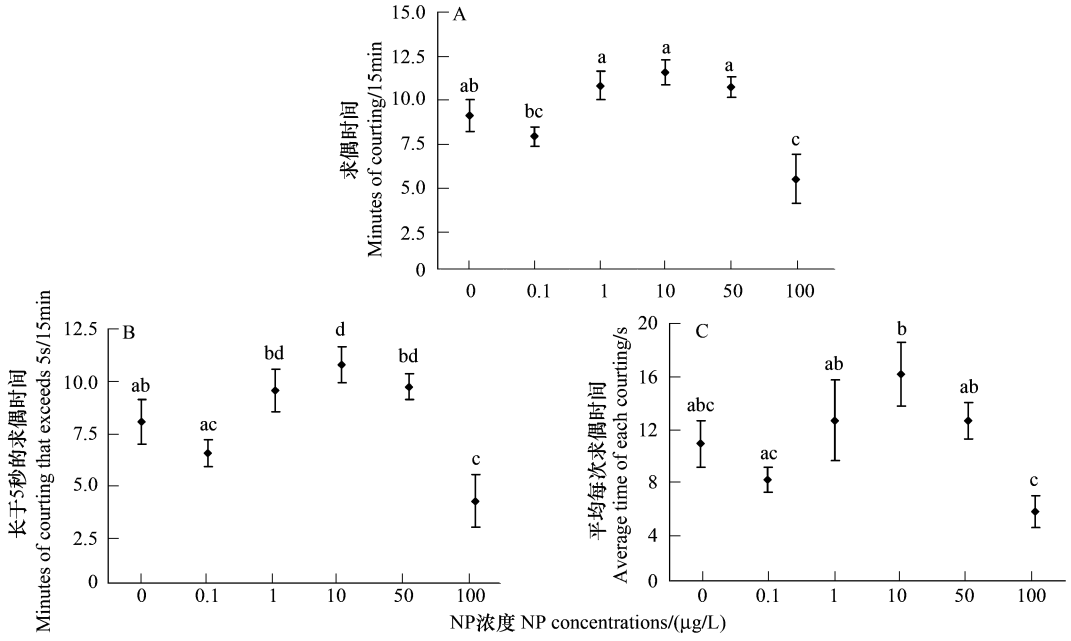

图 1　壬基酚对斑马鱼求偶时间的影响

Fig. 1　Effects of exposure to different NP concentrations on time of courtship behavior in zebrafish (*Danio rerio*)

缺乏共同的上标字母的数值间有显著性差异 ($P < 0.05$)

响显著($P < 0.05$);平均每次求偶时间随 NP 浓度的升高呈现先增加后减少的趋势,然而各 NP 浓度处理组与对照组相比无显著性差异($P > 0.05$)(图 1C)。

NP 暴露对斑马鱼求偶频率、长于 5 s 的求偶频率及短于 5 s 的求偶频率均无显著性影响($P > 0.05$),各 NP 浓度处理组与对照组相比也无显著性差异($P > 0.05$),如图 2 所示。

2.2 繁殖率

NP 暴露对斑马鱼产卵量的影响显著($P < 0.05$),高浓度(100 μg/L)NP 暴露导致斑马鱼产卵量显著减少,其余各组与对照组相比无显著性差异($P > 0.05$)(图 3)。不同浓度 NP 暴露对斑马鱼受精率的影响显著($P < 0.05$),高浓度(100 μg/L)NP 暴露导致斑马鱼受精率显著低于其它处理组($P < 0.05$),低于 100 μg/L NP 暴露对斑马鱼受精率无显著影响($P > 0.05$)(图 4)。

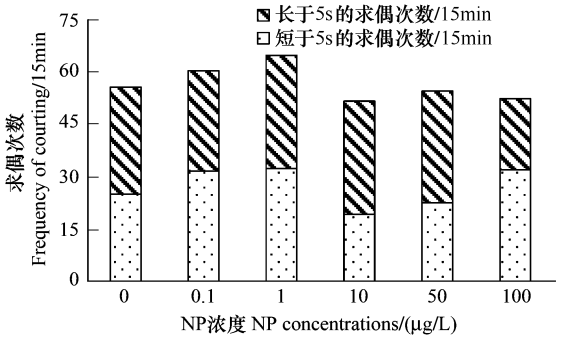

图 2 壬基酚对斑马鱼求偶频率的影响

Fig. 2 Effects of exposure to different NP concentrations on frequency of courtship behavior in zebrafish (*Danio rerio*)

2.3 求偶行为与产卵量的关系

为探明求偶行为与产卵量的关系,对求偶行为与产卵量作偏相关分析,结果见表 1。斑马鱼产卵量与求偶总时间、平均每次求偶时间、长于 5 s 的求偶时间显著正相关($P < 0.05$),与求偶总时间、长于 5 s 的求偶时间相关性较高,相关系数分别为 0.637、0.619。斑马鱼产卵量与求偶频率以及短于 5 s 的求偶频率不相关($P > 0.05$),与长于 5 s 的求偶频率弱相关,相关系数为 0.497。

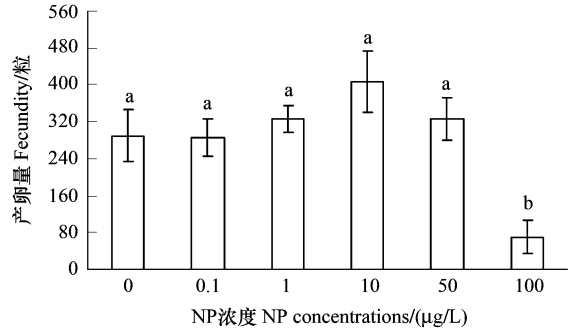

图 3 壬基酚对斑马鱼产卵量的影响

Fig. 3 Effects of exposure to different NP concentrations on fecundity of zebrafish (*Danio rerio*)

缺乏共同的上标字母的数值间有显著性差异 ($P < 0.05$)

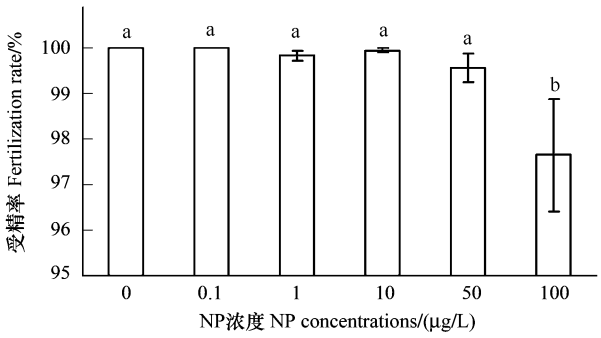

图 4 壬基酚对斑马鱼受精率的影响

Fig. 4 Effects of exposure to different NP concentrations on fertilization rate of zebrafish (*Danio rerio*)

缺乏共同的上标字母的数值间有显著性差异 ($P < 0.05$)

表 1 斑马鱼产卵量与求偶行为的偏相关分析

Table 1 Partial correlation analysis of fecundity and courtship behavior in Zebrafish (*Danio rerio*)

	产卵量 Fecundity					
	求偶总时间 Minutes of Courting	平均每次求偶时间 Average time of each courting	长于 5 s 的求偶时间 Minutes of Courting that exceeds 5 s	求偶频率 Frequency of courting	长于 5 s 的求偶频率 Frequency of courting that exceeds 5 sec.	短于 5 s 的求偶频率 Frequency of courting that less than 5 s
R	0.637	0.414	0.619	0.009	0.497	−0.271
P	0.000	0.004	0.000	0.951	0.000	0.065

控制变量: NP 浓度. *R*: 偏相关系数

3　讨论

NP 是水生生态系统一种常见的环境内分泌干扰物。淡水环境 NP 的浓度范围从低于 0.01 μg/L 到高达 180 μg/L[16]。长期以来,肝脏卵黄蛋白原(VTG)被作为最常用的生物标记用于卵生动物毒理学研究。Van den Belt 等[17]和 Yang 等[18]分别以 VTG 为生物标记研究了 NP 暴露 21 d 对斑马鱼成鱼的毒理学效应,结果发现最低可见效应浓度(LOEC)为 100 μg/L 或更高(500 μg/L)。本文研究结果表明,NP 对斑马鱼求偶行为影响显著,100 μg/L NP 暴露显著抑制求偶行为,说明斑马鱼求偶行为有望作为有效的生物标记用于水体 NP 污染评价[19]。

鱼类性行为的改变是评价 EDCs 对鱼类影响的关键毒理学效应之一[9]。野外研究表明,遭受造纸厂污水污染的溪流中生活的雌性食蚊鱼(*Gambusia affinis affinis*)表现出雄性的性行为特征[20]。1 μg/L 雌二醇(E_2)水体暴露导致金鱼(*Carassius auratus*)追尾时间显著减少[21]。Nakayama 等[22]的研究表明,三丁基锡(TBT)暴露抑制青鳉(*Oryzias latipes*)的性行为,而多氯联苯(PCBs)对青鳉的性行为影响不显著。有人分别用不同的方法研究了 17α-乙炔基雌二醇(EE_2)对斑马鱼雄鱼求偶行为的影响,结果却有所不同,Larsen 等[12]认为高剂量 EE_2(5 ng/L)从卵受精到性成熟的整个生活史暴露抑制斑马鱼成鱼的求偶行为,低剂量的 EE_2(0.05 ng/L,0.5 ng/L)暴露对求偶行为无影响;Colman 等[23]的研究结果则表明,0.5 ng/L EE_2 暴露 48 h 减少求偶行为,5 ng/L 与 50 ng/L 高剂量 EE_2 暴露 48 h 对斑马鱼求偶行为无显著影响。本文中,NP 暴露对斑马鱼求偶总时间、长于 5 s 的求偶时间、平均每次求偶时间的影响显著,高浓度(100 μg/L)NP 暴露导致斑马鱼求偶总时间以及长于 5 s 的求偶时间显著减少,研究结果与 Bjerselius 等[21]和 Larsen 等[12]相一致。NP 暴露对斑马鱼长于 5 s 的求偶时间的影响呈“∩”型毒理曲线,中等浓度(10 μg/L)NP 暴露导致斑马鱼长于 5 s 的求偶时间增加。作者推测,这种现象可能主要归因于 10 μg/L NP 暴露诱使雌鱼表现出积极的性行为,在繁殖行为中表现为比较积极配合、运动速率降低,从而导致雄鱼长于 5 s 的求偶时间增加;在雄鱼的求偶过程中,雌鱼的角色同样不容忽视,EDCs 可能通过作用于雌鱼的生殖内分泌系统,使雌鱼在繁殖行为中表现为不同程度的逃避或是迎合。EDCs 干扰鱼类生殖内分泌系统的途径比较复杂,其主要作用位点可能发生在下丘脑、垂体、性腺、甲状腺和肝脏等任一器官。目前,EDCs 影响动物繁殖行为的机制尚不明确,EDCs 可能直接作用于睾丸或间接通过下丘脑—垂体—性腺轴(HPG)抑制雄激素的合成与分泌,进而抑制求偶行为[24-25],也可能是直接作用于大脑,抑制调控繁殖行为的神经系统[22,26]。此外,求偶行为表现还与雄鱼对雌鱼性信息素的嗅觉反应能力有关[21]。EDCs 暴露可能造成雄鱼对雌鱼性信息素感测能力的下降,进而使雄鱼在求偶过程中表现为兴奋性差,追逐能力下降,最终导致繁殖率降低。

已有的研究结果表明,NP 暴露可导致梭鲈鱼(*Sander lucioperca* L.)产卵量、精子质量以及受精率下降[27],100 μg/L NP 暴露 21 d 降低青鳉产卵量与受精率[28]。Kang 等认为产卵量与受精率下降的原因主要有 EDCs 抑制雄鱼性行为、导致雄鱼精子发生障碍,以及损伤雌鱼生殖内分泌系统等[29]。NP 可抑制睾酮合成,并通过干扰垂体释放促性腺激素间接影响睾丸间质细胞与支持细胞功能,Weber 等报道了 NP 暴露后青鳉睾丸生精细胞、支持细胞和间质细胞的凋亡率显著升高[30]。受精率降低可能是由于 NP 抑制了精子发生或精子释放所致[31],也可能是由于 NP 暴露降低了精子活力与卵子质量所造成的[32]。此外,还有报道称 NP 暴露可抑制花斑溪鳉(*Rivulus marmoratus*)的卵子发生[33]。本文中,NP 暴露显著影响斑马鱼产卵量和受精率,100 μg/L NP 暴露导致斑马鱼产卵量和受精率均显著降低,研究结果与 Popek 等和 Ishibashi 等相一致[27-28]。求偶行为与产卵量偏相关分析结果显示,产卵量与求偶时间(求偶总时间、平均每次求偶时间、长于 5 s 的求偶时间)显著正相关,而与求偶频率关系不显著,说明求偶时间而非求偶频率是影响产卵量的重要因素。NP 暴露可能通过影响斑马鱼求偶时间,进而影响产卵量。

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