アフィメトリクス社のプロトコールに従い実施した。全RNA（およそ100ng）に対し、T7プロモーターの付加したオリゴdTプライマーを用い逆転写しcDNAを得、2本鎖とし、T7 RNAポリメラーゼ（Ambion社）を用いてcRNAを合成（この段階ではビオチン化塩基は用いない）した（増幅1回目）。そのcRNAを鋳型にrandom primerを用いて逆転写してcDNAを得、2本鎖にし、T7 RNAポリメラーゼ（ENZO社）を用い、ビオチン化CTP, UTP共存下cRNAを合成し、断片化した後、GeneChipへのハイブリに供した。

C. 研究結果

本年度は、杉村芳樹班員とのマウス前立腺におけるBPAの作用の網羅的遺伝子発現変動解析を実施中であるのに加え、菅野純班員とのマウス胎児培養神経幹細胞の網羅的遺伝子発現解析を実施した。結果は各班員と共同で解析予定である。

すなわち、

マウス前立腺におけるBPAの作用の網羅的遺伝子発現変動解析（杉村班員）

マウス前立腺における内分泌撹乱化学物質の影響について、Percellome手法を適用したAffymetrix社のGenechipシステムによる網羅的遺伝子発現解析を実施中である。具体的には、杉村班員より送られた細胞サンプルから、RNA抽出を始めとするGenechipシステム解析に必要な反応を行い、データを得、得られたデータを杉村班員と共同で解析予定である。以下でその状況について説明する。

共同研究概要：内分泌かく乱化学物質の前立腺に対する影響の解析を目的とする共同研究を開始した。杉村班員はBPAがマウス前立腺においてエストロゲン様作用を示すことを無血清器官培養法によって確認し、さらにBPAによりTGFaが発現誘導されることを見いだしている。しかし、BPAがTGFaのみを誘導するとは考えられず、網羅的な解析を進めることにより他の因子を検索することが今後の研究方針を立てる上で重要であると考えられた。また、他の動物種ではエストロゲン受容体は間質細胞にのみ発現していると報告されていることを踏まえると、上皮と間質を分けて検討する必要があると考えられる。これらを背景に、具体的な共同研究として、1）BPA経胎盤投与による仔雄マウス成獣時の前立腺変化に関与する遺伝子群の同定、2）成獣雄マウスへのBPA投与に伴う前立腺変化に関与する遺伝子群の同定、3）無血清器官培養法におけるBPAの作用メカニズム解析、の3つのテーマを設定した。これらのテーマを進めるにあたり技術的な課題が浮上した。上皮と間質を分けるために用いる技術であるLCM(laser capture maicrodissection)により得た、極めて微量のサンプルを用いてPercellome手法を

適用した定量的な網羅的遺伝子発現解析を実施するための具体的な方策である。そこで、昨年度導入に成功したT7増幅を2回行うことによる微量サンプル解析法を基本に課題克服に向け検討を加えた。

マウス胎児培養神経幹細胞の網羅的遺伝子発現解析（菅野班員）

胎生11日、12日、13日、14日の胎児終脳からマウス胎児培養神経幹細胞をニューロスフェア培養し、網羅的遺伝子発現解析に供した。胎生中期の神経幹細胞はニューロンにしか分化しないが、後期以降にはグリア細胞にも分化する能力を獲得する。菅野班員により、胎生中期に一時的にDES暴露を受けた後期胎児脳の神経幹細胞がex vivo培養（ニューロスフェア培養）環境下で自己複製不良となることを見出されており、本共同研究はこの現象のメカニズムを探るための基盤サポート研究として実施した。

経時的に網羅的遺伝子発現変動を解析したところ、胎生11日目と12日目では大きな変動は見られないが、13日以降に発現が大きく変動する傾向があることが判明した。変動を示した遺伝子の中には、神経細胞分化に関わるNeurod6やオリゴデンドロサイト分化に関わるOmgに加え、アストロサイトマーカーの一つであるGFAPが含まれていた。すなわち、胎生中期の神経幹細胞に比べ胎生後期の神経幹細胞では、分化に関わる遺伝子の基礎的な発現値が上昇していることが分かった。

D. 考察

昨年度技術導入に成功した微量サンプルの網羅的遺伝子発現解析を基本に、LCMによる極めて微量のサンプルからの網羅的遺伝子発現解析実施について検討を加え、見通しが立った。その結果は杉村班員の今後の研究展開に示唆を与えるものと期待される。菅野班員と行った共同研究からは、胎児発生が進むにつれ神経幹細胞がグリア細胞への分化能力を獲得する現象を反映する遺伝子発現変動が認められた。今後同様の研究に於いて早期に研究の方向が定まり、研究の効率化が図られることが期待される。

E. 結論

網羅的遺伝子発現解析を用いることで、今後各班員の研究方向決定に影響を与える結果が短期間のうちに得られる可能性があることが確認された。

すなわち、網羅的遺伝子発現解析技術は、数万のマーカーを対象に内分泌かく乱候補化学物質の影響を迅速に検討できる有効な技術である。本研究で示されてきているように、この技術は、明確な表現型を示して影響が現れることが少ない内分泌かく乱化学物質の作用を、その作用メカニズムに立ち入って解析する際に本領を発揮するものと期待される。

F. 研究発表

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なし

2. 学会発表

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3. 知的所有権の取得状況

A. 特許取得

なし

B. 実用新案登録

なし

C. その他

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Toxicology and Applied Pharmacology 207 (2005) S617 – S622
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Review

A comparison between integrated risk assessment and classical health/environmental assessment: Emerging beneficial properties

Jun Sekizawa[a,*], Shinsuke Tanabe[b]

[a]*Faculty of Integrated Arts and Sciences, The University of Tokushima, Japan, 1-1 Minamijosanjimacho, Tokushima 770-8502, Japan*
[b]*Center for Marine Environmental Studies, Ehime University, 2-5 Bunkyocho, Matsuyama, Ehime 790-8577, Japan*

Received 15 July 2004; revised 21 January 2005; accepted 21 January 2005
Available online 5 July 2005

Abstract

Both humans and wildlife are exposed to various types of halogenated organic compounds such as polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT), typically old chemicals, and tris(4-chlorophenyl) methane (TCPM) and brominated flame retardants, some new chemicals, simultaneously. Classical risk assessment has evaluated health and ecological risks independently by experts from different disciplines. Taking into considerations the recent concerns about endocrine disrupting chemicals and the progress of research in related areas, we integrated and assessed data on exposure and potential effects in humans and wildlife. Comparisons were made for organ concentrations, body burdens of several organochlorine compounds (OCs), metabolic capacities between humans and various wildlife. When we integrate the knowledge on effects and exposure in humans and in wildlife, new insights were suggested about similarities and/or differences in potential effects among various human populations living on different foods and having different body burdens. Combining existing information with emerging knowledge of mechanisms of actions on endocrine disrupting chemicals after exposure to above chemicals during early developmental stages will further elucidate potential risks from exposure to those chemicals.

Keywords: DDT; *p*, *p*′-DDE; Tris(4-chlorophenyl) methane; PCB; Body burden

Contents

* Corresponding author. Fax: +81 88 656 7263.
E-mail address: sekizawa@ias.tokushima-u.ac.jp (J. Sekizawa).

0041-008X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2005.01.047

Introduction

For practical reasons, human health and environmental risk assessment methodologies have developed independently. However, with increased recognition of the need to protect both humans and the environment more effectively, an integrated approach to risk assessment that addresses real-life situations of multi-chemical, multimedia, multi-route and multi-species exposures is needed. Such an integrated approach would (1) improve the quality and efficiency of assessments through the exchange of information between human health and environmental risk assessors; and (2) provide more coherent inputs to the decision-making process. In response to this need, the UNEP/ILO/WHO, International Programme on Chemical Safety (IPCS), in collaboration with the U.S. Environmental Protection Agency, European Commission, and other international and national organizations, developed a working partnership to foster the integration of assessment approaches to evaluate human health and ecological risks. General framework of the integrated risk assessment was published previously (Suter et al., 2003). To substantiate this approach, an actual demonstration of the integrated risk assessment process is required to facilitate understanding and acceptance of the IPCS framework for integrated risk assessment by risk assessors and risk managers. This study composes a part of the above project to show potential benefits of the integrated risk assessment referring to existing health and ecological assessment documents on DDT and related compounds and to compare them with new approach applying some new data available.

Integration in problem formulation

The first step in the risk assessment process is the problem formulation, which identifies problems, needs of assessment, assessment objectives and the scope of assessment activities as well as the resources available for the assessment. Here, we describe needs for integrated approach with some organochlorine compounds (OCs) on the basis of new concern for endocrine disrupting compounds and recent progress of research in related areas.

Previous evaluations

Both humans and wildlife are exposed to various types of halogenated organic compounds such as PCBs, DDT and its metabolites like 1,1′-(2,2-dichloroethenyldiene)-bis(4-chlorobenzene) or *p*, *p*′-DDE, typically old chemicals, and TCPM and brominated flame retardants, some new chemicals, simultaneously. Classical risk assessment has evaluated health and ecological risks independently, typically assessed and reported by people from different disciplines. There are several international assessment or review documents for DDT and related compounds; however, there are no such documents found for TCPM. TCPM may originate from a variety of sources including production of synthetic high polymers, light-fast-dyes for acrylic fibres, anthelminthic drugs and formulations of agrochemicals, such as dicofol and technical DDT (Jarman et al., 1992). The International Programme on Chemical Safety (IPCS), a leading international body to evaluate chemical safety in terms of health and the environment, has evaluated DDT and its derivatives in two occasions independently, once for health aspects, and in another time for environmental aspects in its Environmental Health Criteria series (IPCS, 1979, 1989). The Joint FAO/WHO Meeting on Pesticide Residues (JMPR), an international expert committee associated with IPCS and also with the Codex Alimentarius, has reviewed toxicological aspects of DDT and its derivatives, several times from 1963 to 2000 (WHO, 2001). In the above IPCS's document for ecological risk assessment on DDT and its derivatives, it was mentioned that there is a fundamental difference in approaches between toxicologists and ecologists concerning the appraisal of the potential threat posed by chemicals (IPCS, 1989). For example, toxicologists are said to be preoccupied with any adverse effects on individuals, whether or not they have ultimate effects on performance or survival; however, ecotoxicologists are concerned primarily with the maintenance of population levels of organisms in the environment.

Impetus for the assessment

Recent concern about endocrine disrupting chemicals and progress of research in related areas has given us impetus to integrate and assess data on exposure and potential effects in humans and wildlife which may share similar exposure pathway and show potential effects to both humans and wildlife through similar mode of actions. Ecotoxicologist is said to be interested in effects on the performance of individuals in their reproduction and survival insofar as these might ultimately affect the

population size. Incidentally, a recent evaluation of DDT and its derivatives by the JMPR 2000 group, took into consideration the hormone modulating effects as one of the relevant effects for its toxicological evaluations.

Some evidence of endocrine disrupting potency of p, p′-DDE and TCPM

TCPM has similar chemical structures with *p, p′*-DDE (Fig. 1) which is known to cause eggshell thinning in pelicans, cormorants and other avian species (Cooke et al., 1976). TCPM showed binding activity to androgen receptors comparable to that of *p, p′*-DDE and the calculated K_i (0.62 μM) for TCPM was lower than reported K_is for antiandrogenic pesticide *p, p′*-DDE and vinclozolin in in vitro studies, although TCPM did not show effects on serum testosterone levels and morphology of testis in an in vivo study. An in vivo study showed that dietary dose of 12.4 mg/kg/day of TCPM to Sprague–Dawley rats elevated follicle stimulating hormone (FSH) in terminal blood samples (Foster et al., 1999).

Potential integration benefits

It might be useful to integrate and exchange information between ecotoxicologists and health toxicologists on the basis of new concern to the endocrine disrupting compounds and recent progress in the research in related areas to better answer needs of the risk managers and the societies. Formerly, the major effects of DDT and related compounds to humans were considered to be on the nervous system which is associated with the effect on the membrane in the nervous system (IPCS, 1979), while their effects to birds such as eggshell thinning (IPCS, 1989), were assessed independently with no consideration of possible links in effects between humans and wildlife. However, recent observations of endocrine disruptive effects of DDE and TCPM suggest that there might be potential risk not only to wildlife, but also to humans originating from common mechanism of actions and common route of exposure to these chemicals. Through integrating information of the health and ecotoxicological research, we may be able to effectively address possible difference and similarities between wildlife and humans.

Integration in the analysis plan

In this step of the risk assessment, one plans how to examine quantitatively the levels of exposure in addition to route of exposure and media for certain chemicals, while on the other hand, data of dose–response relationship for identified effects must be critically examined. New insights on similarities and differences in health and ecotoxicological effects in humans and wildlife will be obtained through integration of information in humans/experimental mammals and wildlife.

Exposure assessment

Identification of the source, environmental fate, exposure levels from various routes and ADME are major components in the exposure assessment step. There are fairly accurate estimates of the daily intake of DDT in several developed countries. Exposure aspects, such as environmental occurrence and fate, and route of exposure, are considered to be fairly common to both humans and wildlife, because food constitutes major route of exposure to DDT-type compounds for organisms of higher trophic levels although intake and kinetics may differ widely between them. In the case of persistent organohalogen compounds which are considered to cause hazardous effects chronically, body burden and tissue distribution may compose important parts in the exposure assessment. Therefore, we focused our approach of the integrated assessment to compare body burdens and tissue distributions in this study.

Tissue distribution and composition of OCs accumulation in humans

Amounts of OCs in adipose depot and in bile of humans were calculated from concentrations in adipose tissue multiplied by the weight of adipose tissue obtained from cadavers of patients in a hospital in Tokyo with

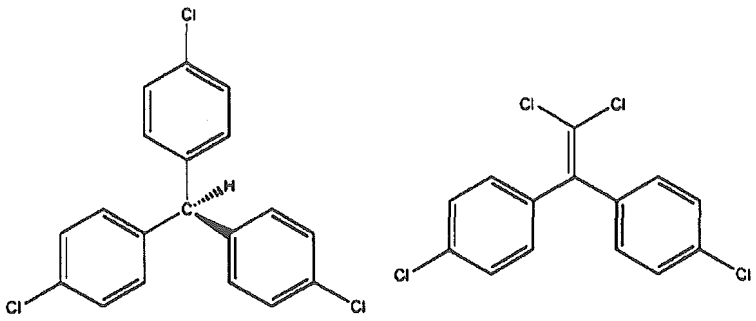

Fig. 1. Chemical structure of 1,1′-(2,2-dichloroethenyldiene)-bis(4-chlorobenzene) (*p,p′*-DDE) (right), and tris(4-chlorophenyl) methane (TCPM) (left).

informed consents from their families (Table 1) (Minh et al., 2001). Weight of adipose tissue (7700 g) was estimated from average body weight of patients (54 kg) multiplied by average fat content of the Japanese people (14.3%). Amount of OCs in bile (ng) was estimated assuming that maximally 1200 g bile is excreted per day, and lipid content of bile is 0.8% to obtain 9.6 g lipid in bile, which is then multiplied by OCs' concentrations in bile. Bile excretion rate is calculated as the ratio of the OC content in the bile to the amount of OCs in the adipose tissue. Since human adipose/bile and liver/bile concentration ratios are fairly close to each other, it suggests equilibrium between these tissues and biliary excretion of OCs (Table 2) (Minh et al., 2001). Low biliary excretion rates especially with *p, p′*-DDE, and TCPM indicated persistent accumulation of these compounds in the human bodies.

Comparison of OC patterns between humans and marine mammals

When compositions of OCs in Japanese human adipose tissues were compared to those in the blubber of cetaceans collected from Japanese coastal waters, humans have higher *p, p′*-DDE in total DDTs, and higher oxychlordane in total chlordanes than marine mammals (Minh et al., 2000; Tanabe, 2002). This suggests that there exists some difference between humans and certain wildlife in that some marine mammals which are devoid of phenobarbital-induced mixed function oxidases (MFOs), and having lower content of methylcholanthrene-induced MFOs, have lower capacities to metabolize OCs.

Potential integration benefits

Organ concentrations, body burdens of several environmentally polluting compounds, and metabolic capacities were compared between humans and various wildlife. Above example illustrates that simple estimation from combination of fat content of the body, octanol–water partition coefficient of the compound, and environmental concentrations is not enough, but integration of other information such as on difference of metabolic capacities among various species is important in explaining large species differences in body burden among species. New insights were suggested about potential differences and similarities in effects not only between humans and wildlife, but also among various human populations living on different foods (see below) and having different body burdens from knowledge thus far integrated regarding OC compounds. This information together with emerging knowledge on mechanisms of actions of endocrine disrupting chemicals after exposure during early developmental stage will further elucidate potential risks to both humans and wildlife from exposure to those chemicals.

Table 1
Concentrations of several organochlorine compounds in human adipose tissue, liver and bile from Japan (ng/g lipid wt)[a]

Sample no.	Fat (%)	TCPM	PCBs	*p, p′*-DDE	DDTs
Adipose tissue					
Mean	63	18	1904	2321	2300
Range	46–76	2.7–44	230–6600	150–7900	160–8100
Geometric mean	65	16	1514	1413	1514
Liver					
Mean	7.3	7	1237	1299	1600
Range	2.5–14	1.1–20	240–2900	120–5600	140–5800
Geometric mean	6.3	5.01	1023	692	891
Bile					
Mean	0.8	17	2871	642	880
Range	0.5–1.5	<5–62	1800–4600	130–1800	160–1900
Geometric mean	0.74	14.5	2754	631	676

[a] Samples were taken from 3 female and 4 male persons.

Table 2
Mean concentrations ratios of organochlorines between adipose tissue, liver and bile in humans from Japan (n = 7)

	TCPM	PCBs	*p, p′*-DDE
Concentration ratio			
Wet weight basis			
Adipose/liver	24	14	14.0
Adipose/bile	130	57	212
Liver/bile	5.5	4.1	15.2
Lipid weight basis			
Adipose/liver	3.19	1.48	2.04
Adipose/bile	1.1	0.54	2.20
Liver/bile	0.35	0.37	1.10

Lipid content in adipose tissue: 635%, liver: 7.3%, bile: 0.8%.

Dose–response assessment

The JMPR group in 1984 evaluation (FAO, 1985) estimated a no-observed-adverse-effect-level (NOAEL) for nongenotoxic carcinogenicity from a rat 2-year study as 6.2 mg/kg bw. per day, and also they concluded then, that there was no firm evidence that DDT has any reproductive or teratogenic effects. They also estimated 0.25 mg/kg bw. per day as an overall NOAEL for humans for no changes in liver functions as observed in workers exposed to DDT of 0.05–0.25 mg/kg bw. per day. However, the 2000 JMPR evaluations (WHO, 2001) noted based on new findings, that activation of estrogen receptors and inhibition of androgen receptors may be mechanisms of the action of DDT-related compounds which led to the observed perturbation of reproductive function. NOAEL of 1 mg/kg bw. per day for developmental effects was estimated based on observations of decreased ovarian weights, cystic ovaries, loss of corpora lutea, infertility, premature puberty, altered onset of vaginal opening, tail anomalies, and increased pup mortality rates. On the other hand, developmental effects, such as eggshell thinning are known to be caused by *p,p′*-DDE (Cooke et al., 1976), and some mammals such as bat which shows marked seasonal cycles in fat content are affected by DDT and its metabolites, although exact mechanisms were not known (IPCS, 1989). Recently, potential effects of

endocrine disrupting chemicals after exposure during critical stages of development to them at low dose levels, which were not known before, were suggested (IPCS, 2002). In addition, body burdens of TCPM, DDT, DDE and PCB on the lipid basis were shown to be sometimes similar between fish-eating human populations and seals or birds, in which seals were collected during outbreak of unusual mortality in Caspian sea caused probably by canine distember virus to immune function deteriorated animals (Minh et al., 2001, Kajiwara et al., 2002, Kunisue et al., 2002).

Potential integration benefits

Old findings of eggshell thinning suggested that this effect might be induced by hormonal disturbance. By the accumulation of knowledge in in vitro and in vivo studies in experimental animals together with supporting evidence from wildlife observations, a new evaluation was deduced. Recent reports (for example, Miyazaki et al., 2004) suggest that some organochlorine compounds and/or their metabolites can exert neurobehavioral or immune effects at very low dose levels. Investigations of this kind of effects which can be induced via disturbance of regulations of the biological process at molecular and cellular levels may change current estimation of risks by these compounds. Here again, we can benefit from the integration in the risk assessment process.

Integration in risk characterization

Formerly, an acceptable daily intake (ADI) of 0–0.02 mg/k bw. was allocated in 1984 for combination of DDT, DDD, and DDE, principally based on human studies where no overall change was observed in liver functions in workers exposed to 0.05–0.25 mg/kg bw., and it was converted to provisional tolerable daily intake in 1994 because of the lack of reliable data on the consequence of exposure to these compounds. However, the JMPR 2000 evaluation derived a Provisional Tolerable Daily Intake (PTDI) of 0.01 mg/kg bw. through its toxicological evaluations on the basis of the NOAEL of 1 mg/kg/day for the developmental toxicity in rats and a safety factor of 100 as described above (WHO, 2001).

Comparison of organochlorine residues in human liver from several countries

Distribution of several OCs in human liver and their ratios were compared (Table 3). PCB concentration is highest in Greenlanders (42,000 ng/g lipid), followed by Norwegians (1900 ng/g lipid), Japanese, Finnish and Swedish with the lowest average concentrations in Americans and Canadians. Very high concentrations in Greenlanders are probably reflecting their food intake pattern where they eat much meat of marine fish or mammals which accumulate high OCs in their adipose tissues. However, compared to PCB concentrations, DDT concentration was highest among Americans and Canadians (3600 ng/g lipid), followed by Greenlanders (2900 ng/g lipid), Japanese, Swedish, Norwegians, and Finish with the lowest average concentrations in Italians (310 ng/g lipid). The PCB/DDT ratios were the highest in Greenlanders (14.4), medium in Finish (2.2), low in Japanese (0.75), and very low in Americans and Canadians (0.08). Since some PCBs (coplanar PCB) are known to exert dioxin-like effects via arylhydrocarbon receptors, or developmental effects possibly via perturbation of thyroid and retinoid metabolism, while *p,p'*-DDE, a metabolite of DDT was shown to exert its effects possibly as an antiandrogen. This wide difference in the amount and the ratio of OCs accumulations among various populations, not only reflects their food intake patterns, but also suggests that OCs may possibly exert different health effects among them. To understand risks better from the exposure to these OCs and for efficient risk management thereof, we need to integrate our knowledge on OC exposure and their possible effects further.

Table 3
Comparison of organochlorine residues (ng/g lipid wt) in human liver from different countries

Country	Year	PCBs	DDT	PCBs/DTT	References
Japan	1999	1023	891	1.15	Present study
Finland	1982–83	1100	550	2.2	Minh et al. (2001)
Norway	1977	1900	800[a]	2.4	ibid.
Sweden	1997	1100	840[a]	1.31	ibid.
Italy	1989	nd	310[b]	–	ibid.
Greenland	1992–94	42,000	2900[c]	14.4	ibid.
US and Canada	1980s	280	3600	0.08	ibid.

Abbreviations: nd, not determined.
[a] *p, p'*-DDE only.
[b] Sum of *p, p'*-DDE, *p, p'*-DDT and *o, p'*-DDT.
[c] Sum of *p, p'*-DDT and *p, p'*-DDE.

Potential integration benefits

We may be able to obtain a better understanding of our exposures and potential effects to both human health and wildlife by integrating our knowledge and information available to us. We need to compare and understand what kind of risk from what types of chemicals via which route of exposure may potentially threat our health and wildlife, and efficiently allocate our resources to better cope with the problems. Integrated risk assessment as shown in this study, will help us judge based on holistic view combined with mechanistic knowledge in the background. Sekizawa et al. (2003) showed another good example of integrated risk assessment using the case of organotin compounds, in which they pointed out that understanding of the basic mechanism behind the apparently independent phenomena in wild life (imposex which is penis development in female gastropods) and experimental mammals (immune toxicity), will elucidate a link between them. Recent report of a

finding (Kaneko et al., 2004) that organotin compounds can be bound with high affinity to retinoid receptors which play important roles in transcriptional regulation of diverse cellular functions, will substantiate this suggestion. A case study for the persistent organic pollutants was published also previously (Ross and Birnbaum, 2003).

Conclusions

Integrated knowledge on chemical properties and on similarities and/or differences in toxicokinetics of endocrine disruptors will give us new insights on potential effects among various populations living on different foods and having different body burdens. Endocrine disruptors are a matter of concern and have impacts on both human health and wildlife. Parallel studies of toxicokinetics and dynamics on humans and wild life, and integrated risk assessment based on them will tell us how and to what extent the environmental pollution is posing risk to human health and wildlife. Integrated risk assessment thus will give us better understanding and consistent basis on effective environmental risk management from both health and environmental protection points of view.

Acknowledgments

This study is a part of the authors' cooperative studies on the Potential risk to future generations from exposures to complex chemical mixtures, supported by the Ministry of the Environment, Japan (2000–2002).

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Chemosphere 61 (2005) 1168–1174

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Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs

Shigeto Oda [a,*], Norihisa Tatarazako [a,c], Hajime Watanabe [b,c], Masatoshi Morita [a], Taisen Iguchi [b,c]

[a] *National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 304-8406, Japan*
[b] *Okazaki Institute for Integrative Bioscience, National Institutes of Natural Science, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan*
[c] *Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation (CREST, JST), Kawaguchi 332-0012, Japan*

Received 29 November 2004; received in revised form 26 January 2005; accepted 22 February 2005
Available online 13 April 2005

Abstract

We exposed the water flea *Daphnia magna* (Cladocera, Crustacea) to either juvenile hormone I (JH I), juvenile hormone II (JH II), or the juvenile hormone-mimicking insecticides kinoprene, hydroprene, epofenonane, or fenoxycarb. By 21-day reproduction tests, we investigated the effects on the number of neonates born per female and the offspring sex ratio. All six chemicals induced *D. magna* to produce male neonates; the male sex ratio of the offspring increased as the chemical concentration increased. EC50 values for production of male neonates were estimated as 400 (JH I), 410 (JH II), 190 (kinoprene), 2.9 (hydroprene), 64 (epofenonane), and 0.92 (fenoxycarb) μg/l. The number of neonates produced was reduced with all chemicals at the concentrations investigated. At the EC50 for male production, five of the six chemicals reduced the reproductive rate to less than 50%; the exception was epofenonane, which caused only a slight reduction in reproductive rate. These results were similar to those obtained for five juvenoids studied previously, one of which was studied here again. There are now 10 chemical substances—all juvenile hormones or their analogs—that are known to induce *D. magna* to produce male neonates. This suggests that juvenile hormone is involved in initiating male production followed by sexual reproduction in *D. magna*, and probably in most cladocerans that exhibit cyclic parthenogenesis.

Keywords: Cladoceran; Epofenonane; Hydroprene; Insect growth regulator; Kinoprene; Sex ratio

1. Introduction

Juvenile hormones are major hormones that are widely distributed in the animal kingdom. Intensive studies have elucidated their endocrine functions, especially in insects and crustaceans (Borst et al.,

* Corresponding author. Tel./fax: +81 29 850 2851.
E-mail address: oda.shigeto@nies.go.jp (S. Oda).

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doi:10.1016/j.chemosphere.2005.02.075

1987; Laufer et al., 1987a,b; Laufer et al., 1993; Nijhout, 1994). Various kinds of juvenile hormones are known, for example, JH 0, I, II, and III, 4-methyl JH I (in insects), and methyl farnesoate (in crustaceans).

In insects, for example, juvenile hormones are secreted by the corpora allata. They play a role in many aspects of development and reproduction: metamorphosis; caste determination in social insects such as ants, termites, and honeybees; the regulation of behavior in honeybee colonies; the polyphenisms of aphids and locusts; larval and adult diapause regulation; vitellogenin synthesis; and ovarian development (Nijhout, 1994).

Cladocerans, known as water fleas, are small crustaceans inhabiting lakes and ponds as zooplankton. They are major constituents of food webs, acting as grazers consuming phytoplankton and, at the same time, as food items for invertebrate and vertebrate predators.

Most cladocerans exhibit cyclic parthenogenesis (Hebert, 1987): under favorable environmental conditions they reproduce without males. In response to some environmental cues, they start sexual reproduction; males appear and females start producing mictic eggs, which will be fertilized by the males. The fertilized eggs are in a state of diapause (resting eggs) and can survive harsh environments. Environmental factors such as short day length, food depletion, and high population density are known to be keys to the initiation of sexual reproduction (Hobaek and Larsson, 1990; Kleiven et al., 1992).

This reproductive system enables the cladoceran population to increase rapidly without males in a favorable environment. On the other hand, a population newly founded from resting eggs, which can endure environmental stress, will be able to adapt to a wide variety of unpredictable environmental conditions over large spatio-temporal scales.

Although several key stimuli that induce cladocerans to start sexual reproduction have been detected, the endocrinological mechanisms behind this have not yet been clarified.

Recent studies, however, have revealed that exposure of adult daphnids to juvenile hormones and their analogs induces parthenogenetically reproducing *Daphnia magna* to produce male neonates (Olmstead and LeBlanc, 2002, 2003; Tatarazako et al., 2003). Five chemicals, all of which have juvenile hormone activity, have been found to be active in male neonate production in *D. magna* (Tatarazako et al., 2003). In addition, daphnids are susceptible to the male-sex determining effects of juvenoids during ovarian egg maturation. (Olmstead and LeBlanc, 2002; Tatarazako et al., 2004). Thus, it is certain that juvenile hormones are associated with the initiation of sexual reproduction (e.g., production of male sex in neonates), although it is not clear what the endogenous juvenile hormone is.

Our aim was to collect more data that supported the hypothesis that juvenile hormones are associated with switching of the reproductive system from parthenogenetic to sexual reproduction in cladocerans. We conducted 21-day reproduction tests in *D. magna* with six juvenile hormones or their analogs (1 of which had been tested before), and assessed the occurrence of male sex in neonates.

2. Materials and methods

The small crustacean *D. magna* (Order: Cladocera) was used. The strain that we used has been maintained for more than 10 years at the National Institute for Environmental Studies (NIES) in Tsukuba, Japan.

The chemical substances used in the study were two insect juvenile hormones, JH I [methyl (2*E*,6*E*)-7-ethyl-9-[(2*R*,3*S*)-3-ethyl-3-methyloxiranyl]-3-methyl-2,6-nonadienoate] and JH II [methyl (2*E*,6*E*)-9-[(2*R*,3*S*)-3-ethyl-3-methyloxiranyl]-3,7-dimethyl-2,6-nonadienoate], and three juvenile hormone analogs (insect growth regulators; IGRs), kinoprene [prop-2-ynyl (*E*,*E*)-(*RS*)-3,7,11-trimethyldodeca-2,4-dienoate], hydroprene [ethyl (*E*,*E*)-(*RS*)-3,7,11-trimethyldodeca-2,4-dienoate], and epofenonane [6,7-epoxy-3-ethyl-7-methylnonyl 4-ethylphenyl ether] (Fig. 1). Fenoxycarb, [ethyl 2-(4-phenoxyphenoxy) ethylcarbamate], which was found to be active in inducing male sex in neonates of *D. magna* in a previous study (Tatarazako et al., 2003) was also studied (Fig. 1). Juvenile hormone I (technical grade, ≈78% pure) and Juvenile hormone II (technical grade, ≈78% pure) were obtained from SciTech Ltd. (Prague, Czech Republic).

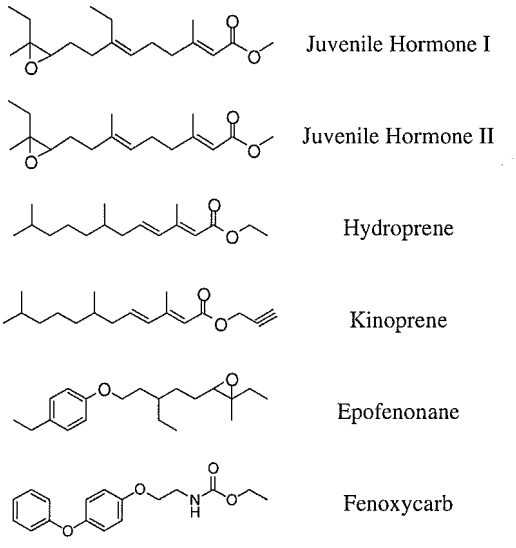

Fig. 1. Chemical structures of juvenoids used in this study.

Kinoprene (96% pure), hydroprene (98.9% pure), and epofenonane (95.0% pure) were purchased from Supelco (Bellefonte, PA, USA), Fumakilla Co., Ltd. (Tokyo, Japan), and Fluka BioChemika (Buchs, Switzerland), respectively. Fenoxycarb (technical grade 96.6% pure) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Experiments were conducted at the nominal concentrations of 62.5–1000 μg/l for JH I, JH II, and kinoprene, 6.25–100 μg/l for epofenonane, 1.25–20 μg/l for hydroprene, and 0.125–2 μg/l for fenoxycarb. Stock solutions of each chemical were prepared at concentrations of 10^1–10^4 mg/l in dimethylformamide (DMF, analytical grade, Wako, Osaka, Japan). Solvent concentrations in all test solutions were less than 0.01% v/v. The culture medium was prepared by filtering tap water through charcoal and keeping it at room temperature overnight.

Experiments were conducted in accordance with the "*Daphnia magna* reproduction test" provided by test guideline 211 of the Organization for Economic Cooperation and Development (OECD, 1998). The sex of neonates was also differentiated as an indicator of endocrine disruption in *D. magna* (Olmstead and LeBlanc, 2002, 2003; Tatarazako et al., 2003).

Daphnids less than 24 h old were used at the start of each experiment. They were exposed to various concentrations of test chemicals and observed and fed daily for 21 days. Cultures were kept in an incubator with the temperature kept at 21 ± 1 °C and a photoperiod of 16-h light/8-h dark. Five concentrations of each test chemical and the control were prepared. For the control, culture medium was prepared by dechlorinating tap water through charcoal at room temperature no more than 24 h before use. Each concentration was prepared by diluting stock solutions with fresh culture medium. Ten replicates were prepared for each concentration, each consisting of an individual daphnid in a glass jar (100 ml) filled with 50 ml of test media. Sixty glass jars and daphnids were therefore used to test each chemical. These jars were tightly closed with Teflon caps to minimize test chemical volatilization.

Each jar was provided with *Chlorella* as food at a concentration of 4.3×10^5 cells/ml daily. Water hardness was 80 mg/l, pH 7.0–7.5, and dissolved oxygen concentration 80–99%.

Test animals were transferred to new medium every 2 days. Neonates were removed from the jar daily, and the numbers of neonates were counted. The removed neonates were raised in 100-ml beakers for several days before sexing. Under a microscope, neonates were examined for sex differentiation by the length and morphology of the first antennae (Olmstead and LeBlanc, 2000). The offspring sex ratio was defined as the ratio of males to the total number of neonates observed at each concentration.

2.1. Statistical analyses

To test the effect of the chemicals on reproduction, analyses of variance (ANOVAs) were performed on the number of neonates born per female over 21 days. When any significant ($P < 0.05$) effect was detected, significance of difference from the control was tested further by Dunnett's test with a P-value of 0.05 as the significance level (StatView, SAS Institute, 1998).

The effect of test chemicals in inducing the production of male neonates was tested by logistic regression analysis, and the significance of regression coefficients was tested by the R stats package (Ihaka and Gentleman, 1996). The median effective concentration (EC50) for male neonate production was also estimated by this analysis.

To evaluate the effect of changes in offspring sex ratio on reproduction or on population growth rate, number of neonates born per female during the 21 days or the intrinsic rate of natural increase (r) were compared for two scenarios, one in which male neonates were included in the data, and the other in which only female neonates were used for calculation. The intrinsic rate of natural increase was calculated by the stable-age (Euler's) equation. These comparisons were made for fenoxycarb and epofenonane. These two chemicals were chosen because typically they have different concentration–response curves for total reproduction and for offspring sex ratio.

3. Results

All six chemicals induced the production of male neonates. The estimated EC50s for male neonate production from logistic curves fitted to the concentration-dependent responses were as shown in Table 1. The highest EC50s among six chemicals were for JH I and II—about 430–450 times as high as that for fenoxycarb, which was the lowest among the six chemicals (Fig. 2, Table 1). However, the low purity of JH I and II (at about 78%) should be taken into consideration.

At about the test concentrations at which male neonates appeared at a rate of 50% (EC50), the number of

Table 1
EC50s for production of male neonates, as estimated by logistic regressions for six juvenoids, with 95% confidence intervals (CI)

Chemical	EC50 (μg/l)	95% CI (μg/l)
JH I	400	380–420
JH II	410	390–430
Kinoprene	190	170–200
Hydroprene	2.9	2.7–3.1
Epofenonane	64	62–66
Fenoxycarb	0.92	0.88–0.96

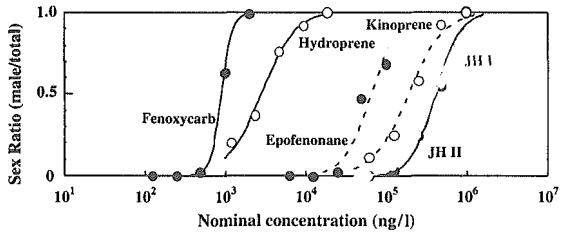

Fig. 2. Sex ratios of offspring exposed to juvenoids. Logistic curves were fitted by logistic regression analysis. Regression coefficients for slopes were all statistically significant ($P < 0.01$).

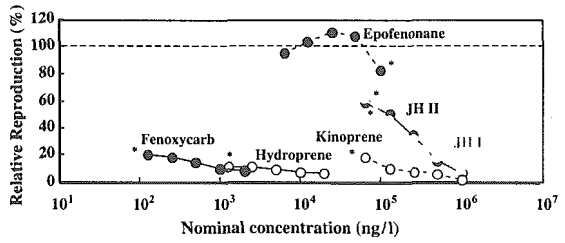

Fig. 3. Mean numbers of neonates born per female over the 21 days are represented as values (%) relative to those of the control group in each experiment. Dotted line represents mean number of neonates per female in the control group in each experiment. Asterisks indicate the lowest significant difference from each control (Dunnett's test, $P < 0.05$).

offspring born over the 21 days was reduced significantly by exposure to all of the chemicals except for epofenonane (Figs. 2 and 3). With epofenonane, in contrast, it was not until the rate of male sex induction reached 69% that the number of neonates significantly decreased (Figs. 2 and 3). A significant decrease was observed only at the highest concentration, at which the total reproduction rate was reduced to about 80% of that of the controls.

This characteristic relationship observed in epofenonane between reduction in reproduction and change in offspring sex ratio with increase in chemical concentration can also be seen with fenoxycarb in the comparison of two scenarios, one in which male neonates were included in the data, and the other in which male neonates were excluded (Figs. 4 and 5). At its lowest concentration (0.13 μg/l), fenoxycarb dramatically decreased the numbers of individuals counted under both scenarios by about 80% of that of control. A significant effect of not including male neonates in the calculation of the number of neonates first appeared at 1.0 μg/l (Fig. 4a). A similar pattern was shown in the intrinsic rate of natural population increase (Fig. 4b). With epofenonane, in contrast, an exceptionally slight reduction in the number of neonates compared with the change in offspring sex ratio was reflected in the results of the comparisons with and without male neonates (Fig. 5a and b). A statistically significant reduction in reproduction compared with the control occurred at a concentration of 100 μg/l, but the reduction was only by about 20% of the control numbers (Fig. 5a). The contribution of the change in neonate sex ratio to the reduction in the number of neonates born was larger: At 50 μg/l, the total reproduction calculated without male neonates was about half that calculated with males (Fig. 5a). This pattern was also shown in the intrinsic rate of natural population increase (Fig. 5b).

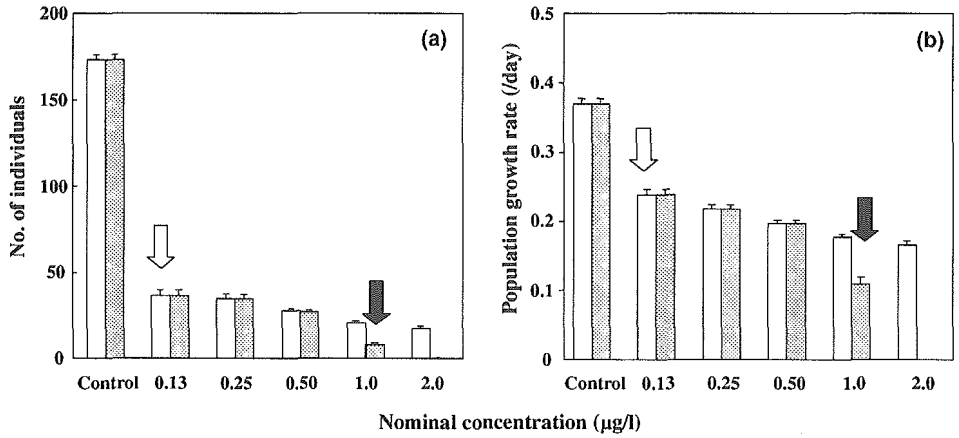

Fig. 4. (a) Mean number of neonates born per female during the 21 days of the test and (b) intrinsic rate of natural population increase calculated for *D. magna* exposed to fenoxycarb. Open bars indicate values calculated by including male neonates, whereas shaded bars show those estimated without male neonates. The lowest nominal concentrations at which a statistically significant decrease from that of the control group was observed are indicated by open arrows. The solid arrows show the lowest nominal concentrations at which there were statistically significant differences between the two calculation methods (including male neonates and excluding them). Error bars indicate standard errors.

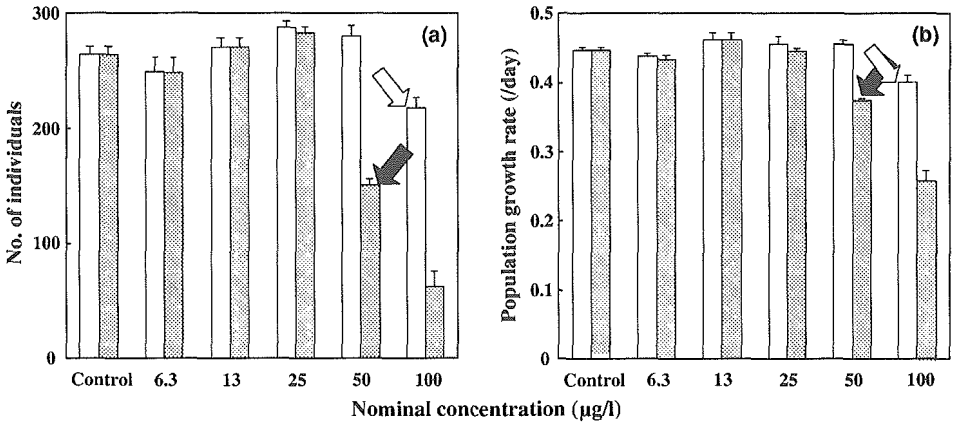

Fig. 5. (a) Mean number of neonates born per female during the 21 days of the test and (b) intrinsic rate of natural population increase calculated for *D. magna* exposed to epofenonane. Open bars indicate values calculated by including male neonates, whereas shaded bars show those estimated without male neonates. The lowest nominal concentrations at which statistically significant decreases from those of the control group were observed are indicated by open arrows. Solid arrows show the lowest nominal concentrations at which there were statistically significant differences between the two calculation methods (including male neonates and excluding them). Error bars indicate standard errors.

4. Discussion

We found that two juvenile hormones, juvenile hormones I and II, and three analogs, kinoprene, hydroprene, and epofenonane, induced *D. magna* to produce male neonates. If we combine our results with those obtained in the previous study (Tatarazako et al., 2003), there are now 10 chemicals known to induce *D. magna* to produce males. All of them are juvenile hormones in insects or crustaceans, or their analogs formulated to mimic their activity for pest control. Although the evidence is only circumstantial, it seems very likely that juvenile hormones play major roles in sex determination in *D. magna*, and probably in a wider taxonomical group of the order Cladocera, too. The mechanism, however, has not yet been elucidated.

Extensive research has been done to address endocrine disruption in crustaceans (e.g., McKenney and Celestial, 1996; Fingerman et al., 1998). In most cases, however, reduction in reproduction rate is adopted as an endpoint. It is difficult to distinguish between toxicity in general and endocrine disruption as the main causes of reduced reproductive rate. It is interesting that all juvenoids brought about the same consequence in the case of *D. magna* (i.e., induction of male sex), despite the widely varying effective concentrations. The use of offspring sex ratio in daphnids as a new endpoint will facilitate the detection of endocrine disruption associated with juvenile hormones, compared with other endpoints such as reproductive rate or molting inhibition, by which endocrine-disrupting effects cannot be detected clearly because of confusion with toxicity. Thus, testing methods that use *D. magna* are suitable for screening of juvenile-hormone disrupting effects.

Daphnia is not suitable for detecting distortions in the sex ratio caused by the endocrine-disrupting effects of chemicals because, unlike many other crustaceans, most cladocerans exhibit asexual reproduction (parthenogenesis) under laboratory conditions. In addition, it is difficult to include mating processes associated with sexual reproduction in the test. Some studies have tried to detect the endocrine-disrupting effects of chemicals in daphnids by using offspring sex ratio as an endpoint (Dodson et al., 1999; Peterson et al., 2001; Olmstead and LeBlanc, 2003). In most cases, the endocrine-disrupting effects of chemical substances are tested by observing the sex of neonates born to the test animals, which have been manipulated to produce both male and female neonates by controlling one or more experimental conditions, such as photoperiod, population density, or food concentration. By such experimental manipulation, it might be possible to identify substances that have "masculinizing" or "feminizing" effects. However, even if the offspring sex ratio of *D. magna* exposed to some chemical deviates significantly from that in a control group, it does not necessarily mean that a "masculinizing" or "feminizing" effect has been detected. This is because the chemical might only have synergistically or antagonistically modified an environmental effect, which was itself the main cause of male sex induction. In our study, no male neonates were found in the control group. Therefore, if confounding effects are removed, *Daphnia* is useful in studies of endocrine-disrupting chemicals and would be an ideal screening system for endocrine-disrupting effects.

The reduction in reproductive rate at the concentration at which 50% of male neonates appeared was less

than about 20% for five of the chemicals, the exception being epofenonane (Figs. 2 and 3, Table 1). A similar relationship was also observed in our previous study (Tatarazako et al., 2003, Figs. 2 and 3). For epofenonane, in contrast, male neonates appeared at a relatively low concentration compared with the concentration at which reproductive inhibition occurred (Figs. 2 and 3). These differences between epofenonane and the other juvenoids are reflected in Figs. 4 and 5. With fenoxycarb, for example, both the number of neonates per female and the population growth rate decreased significantly compared with the control at a concentration of 0.13 μg/l, both with and without inclusion of male neonates in the data (Fig. 4a and b). A significant difference between the results of calculation with and without male neonates was observed at a concentration of 1.0 g/l, which is about 10 times as high as 0.13 μg/l (Fig. 4a and b). This means that it was not the decrease in female neonates caused by the change in offspring sex ratio but the decreased number of neonates itself that contributed most to the reduction in population growth rate at low concentrations.

On the other hand, with epofenonane, a slight reduction in the number of neonates, along with a change in the offspring sex ratio, resulted in a large difference in the calculation of number of neonates and population growth rate with and without inclusion of male neonates (Fig. 5a and b). This is mainly because the decrease in both number of neonates and population growth rate can be attributed to the change in offspring sex ratio.

It is not clear whether the reduction in reproductive rate after exposure to juvenoids was caused by toxicity in general or toxicity through endocrine disruption. The exceptional result obtained for epofenonane suggests that the two effects of juvenile hormone exposure on daphnids—induction of male neonate production and reduction in reproductive rate—are not tightly associated with each other, implicitly negating the possibility of toxicity through endocrine disruption as a major cause of reduction in reproductive rate.

No chemical substance other than juvenile hormones or their analogs has ever been found to be active in inducing male sex in neonates of *D. magna*. With the wide range of juvenile hormone functions in arthropods, more attention should be paid to the application of IGRs. Some juvenoid IGRs are used to exterminate pests on pets or to control pests in greenhouses under controlled usage; others are used unrestrictedly in the field to control or eradicate pests and insects that affect humans and livestock, or in the storage of agricultural products. In this last case, application of the juvenoid IGRs pyriproxyfen or methoprene to control the larvae of dipteran insects such as mosquitoes (Estrada and Mulla, 1986) or chironomids (Ali et al., 1993), for example, could affect some non-target species, such as cladocerans in small ponds.

Some IGRs are specific to target species, but others are less specific and could be active in non-target species. From the results obtained here and in a previous study (Tatarazako et al., 2003), all 10 juvenile hormones and analogs are effective in inducing male sex in neonates of *D. magna*. In addition, not only in *D. magna* but also in four other species (*Moina macrocopa*, *Moina micrura*, *Ceriodaphnia dubia*, and *Ceriodaphnia reticulata*) of cladoceran, male sex in neonates is induced by exposure to the juvenoid IGR fenoxycarb (Oda et al., 2005). This suggests that induction of male neonate production is a common response to juvenoids and that the juvenoids are capable of initiating sexual reproduction in cladocerans, most of which exhibit cyclic parthenogenesis. Because freshwater cladocerans play a major role in aquatic communities, IGRs should be applied carefully. More extensive research addressing the effects of IGRs on non-target organisms is needed.

Acknowledgments

We thank Ryoko Abe and Kazushige Nishizuka for their assistance in the laboratory. This work was in part supported by a Grant-in-Aid for Scientific Research, Priority Area (A), from the Ministry of Education, Sports, Culture, Science and Technology of Japan and by grants from the Ministry of Environment, Japan.

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Shigeto Oda got his M.S. degree in biology at Kyushu University, Fukuoka, Japan in 1995. He finished the doctoral course in biological sciences at University of Tsukuba, Ibaraki, Japan in 2000, where he majored in animal ecology. Since May 2002, he has been conducting research on endocrine-disrupting chemicals at the National Institute for Environmental Studies, Ibaraki, Japan.