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AQUATIC ORGANISMS ON THE PILL

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EFFECTS ON DIFFERENT ORGANIZATIONAL LEVELS

AQUATIC ORGANISMS ON THE PILL

—
EFFECTS ON DIFFERENT ORGANIZATIONAL LEVELS

PER HALLGREN



LUNDS
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AKADEMISK AVHANDLING
som för avläggande av filosofie doktorsexamen vid
naturvetenskapliga fakulteten, Lunds universitet kommer att
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Fakultetens opponent: Dr Iain Barber,
Department of Biology, University of Leicester,
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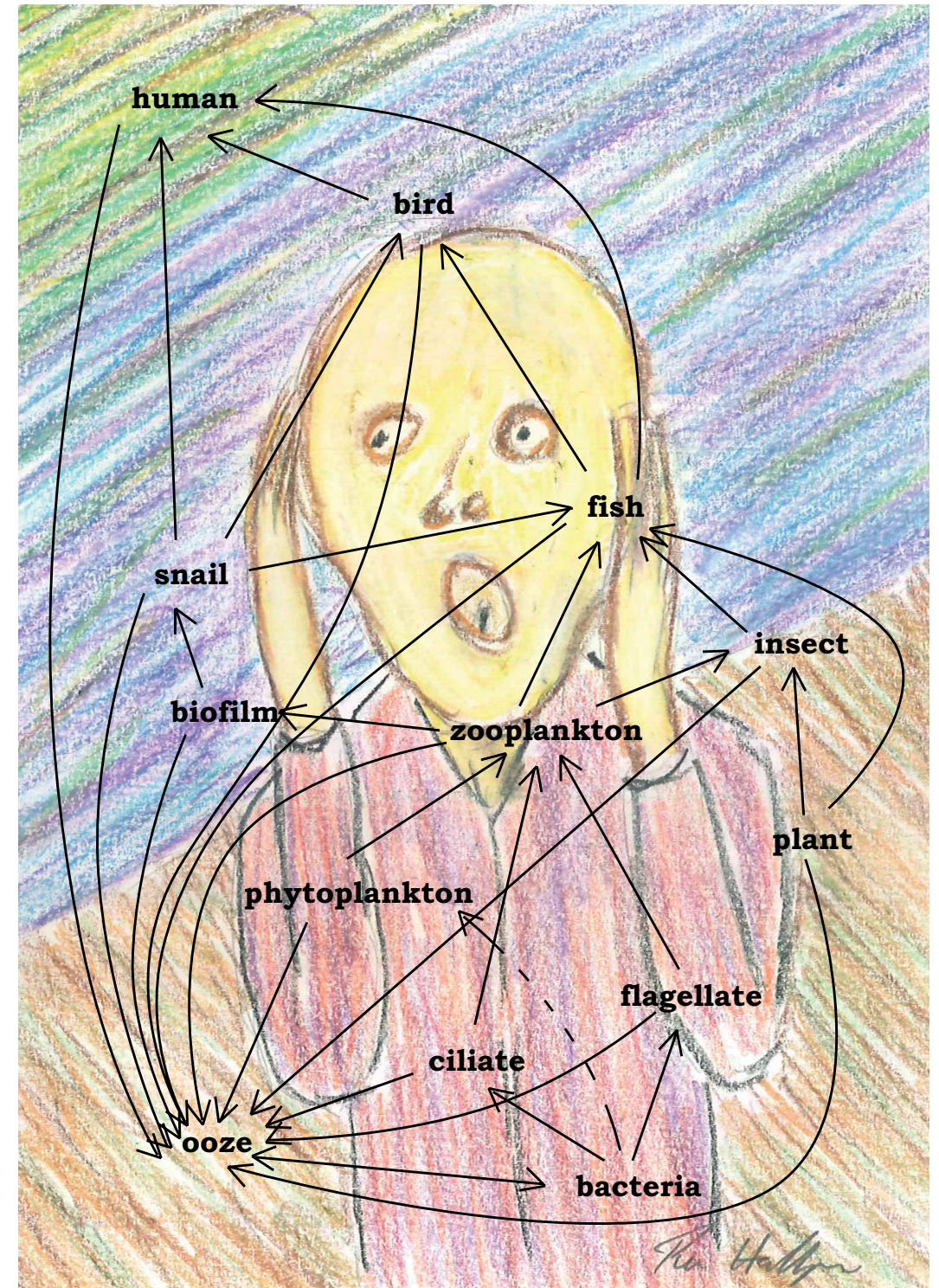
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Abstract Communities are composed of organisms that interact with each other and with the non-biological environment. There are also anthropogenic pollutants in the environment that also interact with the organisms. Estrogenic endocrine disrupting compounds, for example 17 α -ethinylestradiol (EE2, the active compound in contraceptive pills), released into surface waters can have negative effects on the organisms living there. Fish are sensitive to estrogenic EDCs and exposed fish can be less fertile and have inhibited reproductive behaviors that may lead to reduced population sizes while the majority of invertebrates are not sensitive, with the exception of mollusks. With the difference in sensitivity is likely that there may be consequences for aquatic communities, such as changes in the structure and dynamics. We have studied the effects of EE2 on different organizational levels in the aquatic community and with the emphasis on end-points not connected to reproduction. We have shown that fish exposed to EE2 had inhibited somatic growth, increased size of liver, decreased size of gonads and also reduced foraging performance that indirectly affected prey communities by releasing them from predation. We have also shown that EE2 can inhibit or enhance somatic growth rates in gastropods depending on species, but population growth rates was not affected, and that EE2 pollution history in gastropods may also influence the effects of novel stressors. Furthermore, we showed that EE2 can interfere with detoxification and apoptosis in copepods (crustacea) and this can make them more sensitive to novel stressors and affect molting and population demography. The effects of EE2 we have observed on the organisms in our studies, together with the known negative effect on fish reproduction, may have consequences for community structures and ecosystem functioning in surface waters that receive EE2 or other estrogenic EDCs and.			
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- II. Hallgren, P., Nicolle, A., Hansson, L-A., Brönmark, C., Nikoleris, L., Hyder, M., Persson, A. Synthetic estrogen directly affects fish biomass and may indirectly disrupt aquatic food webs. *In press*, *Environmental Toxicology & Chemistry*
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- V. Hallgren P., Nilsson, A., Berglund, O., Persson, A. Assessing the cost of exposure history of *Radix balthica* to 17 α -ethinylestradiol when exposed to the novel stressor cadmium. *Manuscript*
- VI. Souza, M. S., Hallgren, P., Balseiro, E., Hansson, L. A. (2013) Low concentrations, potential ecological consequences: Synthetic estrogens alter life-history and demographic structures of aquatic invertebrates. *Environmental Pollution* 178: 237-243

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SAMMANFATTNING PÅ SVENSKA

Det finns många kemikalier i vår miljö där vissa har visat sig göra hanliga fiskar honliga, äggskalen tunnare hos fåglar och som kan göra honliga snäckor hanliga, och det verkar ju inte vara en bra framtid för dessa djur. Sådana kemikalier går under benämningen endokrina störare, eftersom de stör naturliga biologiska processer som är viktiga för djurs utveckling av organ, homeostas, beteenden vid fortplantning och fertilitet. Kemikalierna kan komma ifrån industrier, besprutning av odlingsmarker eller från avloppsreningsverk där en del hamnar i vattendragen och blir tillgängliga för de djur som lever eller som hämtar sin föda där. Listan över dessa kemikalier är lång men några exempel är polyklorerade bifenyler (PCB) som har använts som brandskyddsmedel i transformatorer, tributyltenn (TBT) som finns i båtbottnfärg, ftalater som används som mjukgörare i plastprodukter, bisfenol A (BPA) som används som hårdgörare i plastprodukter samt 17α -ethinylestradiol (EE2) som är den aktiva substansen i p-piller.

Dessa kemikalier kan störa biologiska processer eftersom de liknar djurens kroppsegna kemiska signalsubstanser, t.ex. hormoner hos ryggradsdjur eller neuropeptider hos ryggradslösa djur. Det råder nog inga tvivel om att dessa kemikalier inte är hälsosamma för djur, eller för människan, och projekt har mynnat ut i utvärderingar från t.ex. världshälsoorganisationen (WHO, World Health Organisation) om hur man skall bemöta dessa problem. Detta kan göras genom en bättre förståelse hur dessa ämnen påverkar miljön, metoder för att övervaka, mäta och utvärdera effekterna samt identifiera nya kemikalier som kan störa det endokrina systemet.

EE2, ett syntetiskt östrogen och den aktiva substansen i p-piller, har visat sig främst påverka ryggradsdjur, t.ex. fiskar och groddjur. Detta beror på att dessa djur ur ett evolutionärt perspektiv inte skiljer sig så mycket från oss och har liknande kroppsegna signalsubstanser och receptorer för dessa ämnen. Den forskning som har bedrivits har visat att EE2 påverkar fiskars könsutveckling, fertilitet och parningsbeteenden på ett sätt som kan vara negativt för hela populationer av fisk. Forskningen har även visat att blötdjur, musslor och snäckor, påverkas av EE2 men graden av påverkan och hur de påverkas är artberoende. Vad som gör snäckor känsliga är dock inte helt klarlagt men det finns indikationer på att de har kroppsegna substanser som liknar de som ryggradsdjuret har, även om de inte är närbesläktade med dem. Andra ryggradslösa djur som insekter och kräftdjur är dock inte känsliga för EE2 i de koncentrationer som finns i våra vattendrag. Att säga att ett djur inte påverkas av EE2 kan dock vara fel, det beror mer på mängden av EE2 som djuret exponeras för (*Allt är ett gift, och ingenting är utan giftighet. Enbart dosen bestämmer att något inte är ett gift.* Paracelsus 1493-1541) samt vad man mäter hos ett djur, på molekylär-, organ-, individ-, eller populationsnivå.

I min forskning har jag försökt att besvara frågor om hur EE2 påverkar populationstillväxten hos snäckor, hur EE2 påverkar fiskars beteenden då de söker föda, om EE2 kan påverka djur som inte är känsliga för EE2 genom indirekta effekter (trofiska kaskader) samt hur fiskars fysiologiska hälsa påverkas. Jag har även studerat om snäckors EE2 exponeringshistoria kan göra dem mer eller mindre toleranta för andra kemikalier samt om EE2 kan ha en påverkan på fysiologiska processer hos kräftdjur som kan påverka deras utveckling, programmerad celldöd, avgiftning och nervsignalering.

De resultat som jag har fått genom min forskning visar på att olika arter av snäckor kan ha olika tillväxt då de är exponerade för EE2, men effekterna var inte förmodligen inte stora nog att påverka populationstillväxten. Vad som skiljer de två arterna av snäckor är att en art är hermafrodit (tvåkönad) och en är separatkönad art. Om skillnaderna i deras tillväxt beror på att de har olika fortplantningsstrategier är dock inte klarlagt, men det visar på att närbesläktade arter kan reagera olika.

Jag observerade också att fiskars beteende då de söker föda kan påverkas negativt vilket försämrar deras födointag och tillväxt. Detta kan förmodligen bero på att fiskarna var exponerade för EE2 under tidig utveckling och då finns det en risk för att utvecklingen av organ kan påverkas negativt. Fiskar som är fullt utvecklade och är vuxna visade inget förändrat födointag, även om deras tillväxt var sämre, levern blev större hos både hanar och honor, samt gonaderna blev mindre hos hanliga fiskar. Ökningen av storleken på deras lever beror förmodligen på att även hanliga fiskar började producera vitellogenin, som är ett ägguleprotein. Förminsningen av gonaderna hos hanar kan påverka deras förmåga att fortplanta sig genom eftersom mindre mängd spermier kan leda till minskad fertilitet. Orsaken till deras lägre tillväxt kan vara en kombination av att energi har gått till avgiftningsprocesser och till en ökad produktion av ägguleprotein samt att EE2 kan förändra aktiviteten hos tillväxthormoner. Att de hade sämre tillväxt kan vara alarmerande då det är viktigt för fiskar att lagra energi inför tider med mindre födotillgång samt att tiden till reproduktion kan förlängas.

Då vi exponerade två olika populationer av snäckor för EE2, där en population hade varit exponerat för EE2 tidigare i sitt liv och den andra hade inte varit exponerat, såg vi att snäckorna som hade varit exponerade för EE2 tidigare hade en högre tillväxthastighet. Vi kan däremot inte med säkerhet härleda den snabbare tillväxten till EE2 exponeringen eftersom populationerna växt upp under olika betingelser även i andra avseenden än EE2 exponering. Förutom detta så såg jag att snäckorna växte sämre om de var utsatta för både EE2 och kadmium. Vi såg även att EE2 kan påverka avgiftningsmekanismerna, programmerad celldöd och nervsignalering hos kräftdjur och detta kan leda till förändrad populations dynamik genom att de inte kan bryta ner gifter sig samt att utvecklingen till vuxna individer fördröjs.

Även om effekterna av EE2 på de organismer, populationer och samhällen som jag har studerat inte verkar vara stora så kan det på lång sikt förändra hur samhällena ser ut i de vattendrag som är påverkade av endokrina störare.

INTRODUCTION

First of all, studies on the effects of endocrine disrupting compounds (EDCs) is not a new subject and “Our stolen future” which is a part of the title from a book written by Colborn et al. (1997) summed up many years of collective knowledge about the effects EDCs had on the health of the environment and the inhabitants living there, including us, *Homo sapiens* (Toppari et al. 1996). The consequences of EDC exposure have been studied in different compartments of the biosphere, in different groups of animals and both in males and females. Eggshell thinning in birds (Elliott et al. 1996), abnormal gonads in alligators (Guillette et al. 1994), feminization of male fish (Harries et al. 1997) and masculinization of female mollusks (Oehlmann et al. 1996) linked to polychlorinated biphenyl (PCB), dichlorodiphenyltrichloroethane (DDT), 17 α -ethinylestradiol (EE2) and tributyltin (TBT) respectively, are just a few, but classical examples illustrating the negative consequences such compounds may have on organisms.

What might be a common aspect linking these examples is that these animals live in, or consume food that live in the aquatic sphere. Water surrounds the organism living there and is also a media for transport of all sorts of chemicals, via rain, drainage and sewage, to streams, rivers, lakes and finally the ocean. This can make life difficult for many of the inhabitants in the aquatic sphere and there is seldom a way for the organisms to escape this,

they just have to try to adapt, evolve and continue to live there.

Secondly, what are EDCs ? In a report from WHO/IPCS (2002) they define them as:

- “An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.”
- “A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.”

There are a few compounds of mainly anthropogenic origin (Colborn et al. 1993; Sumpter 2005) that fits to that definition as they have similar properties to endogenous hormones or neuropeptides and thereby may interact with endogenous receptors (Tsai and Omalley 1994; Lafont and Mathieu 2007) and disturb the endocrine system if taken up by an organism. There are also natural compounds, estradiol and phytoestrogens, which are excreted or released from animals and plants, respectively, that may have similar effects (Kurzer and Xu 1997; Kolodziej et al. 2004).

Depending on their properties, EDCs are may be divided into androgenic, anti-androgenic, estrogenic

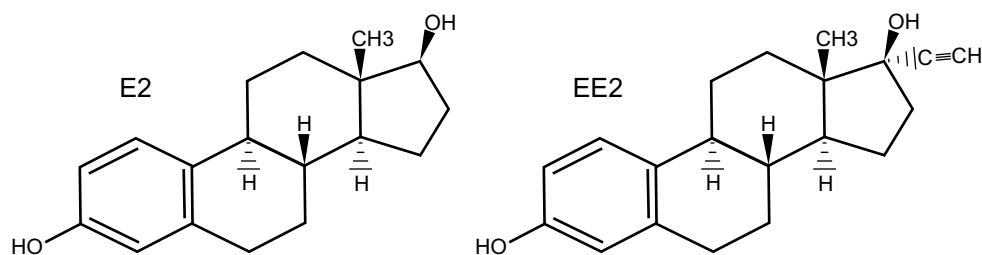


Figure 1. The main character of the story, 17 α -ethinylestradiol (EE2) on the right side, mimicking its natural counterpart, 17 β -estradiol (E2).

and anti-estrogenic. In for example vertebrates, androgens and estrogens regulate the development of male and female characteristics, respectively, by binding to receptors. If the animal is exposed to an EDC, anti-androgenic or anti-estrogenic compound block the receptors and thereby inhibit a process while androgenic or estrogenic compounds bind to the receptor and induce a process, that the endogenous hormone normally would regulate (Sumpter 2005).

There are also EDCs that do not act upon the receptors but may interfere with the endocrine system by other means, e.g. tributyltin (TBT) used in antifouling paints for boats that interfere with the transformation of testosterone to estrogen (Sumpter 2005). Other sources for EDCs can be phthalates (Staples et al. 1997) used in plastic products to make them more flexible and bisphenol A (BPA) (Staples et al. 1998) also used in plastic products but to make them more rigid.

In my research I have studied the effects of 17 α -ethinylestradiol (EE2) (Figure 1), the active compound in contraceptive pills and in hormone replacement therapies and a potent estrogenic EDC (Thorpe et al. 2003) (Table 1), on different organizational

levels of the aquatic community. When EE2 is used the main part is metabolized in the body of the user, but a fraction is excreted in original form with the urine. This means that the majority of EE2 is distributed from urban areas through sewage treatment plants where it is partly degraded (Ternes et al. 1999; Desbrow et al. 1998) and distributed onward to streams, rivers, lakes (Belfroid et al. 1999) and in some cases, the ocean (Pojana et al. 2007; Beck et al. 2005).

So, after almost 20 years after the publication of “Our stolen future” plus approximately 17200 more scientific articles about the subject of EDCs and their effects, have the problems threatening our future disappeared?

No, I do not think so; I just think that we can add new compounds to the already long list of compounds that possess properties that may disturb the endocrine system and the science will continue and try to unravel the possible problems they might cause. However, we can reduce the spread of EDCs, and other chemicals, to our surface waters by enhancing the biological breakdown in sewage treatment plants, inform to the public about how to discharge unused chemicals in a proper way, and use other chemicals that may be less damaging to the environment.

Furthermore, EE2 or other estrogenic EDCs can only induce effects if the individual, either by food, through epidermis or gills, takes up the compound. The compound must also be transported through tissues and either attach at a receptor on the cell wall or within the cell nucleus (Figure 2). The following response may be at molecular level, for example interfering with apoptosis, neurotransmission and detoxifying processes in copepods (Paper VI), liver and gonad size in fish (Paper III and IV), enhanced or inhibited somatic growth in snails (Paper I) inhibited somatic growth in fish (Paper II, III and IV), inhibited or non affected foraging behavior in fish (Paper II and III), population size and structure in zooplankton (Paper II, IV and VI) and size and composition of the community or ecosystem (Paper II and IV).

Table 1. Estrogenic potency for some natural and synthetic estrogenic EDCs relative to the natural estrogen 17 β -estradiol (E2) (Vajda et al. 2008; Metcalfe et al. 2001; Murk et al. 2002; Korner et al. 1998; Korner et al. 2001)

Origin	Compound	Potency
Natural	17 α -estradiol (17 α)	1*10 ⁻³ - 1,1*10 ⁻¹
	Estrone (E1)	5.6*10 ⁻² - 8*10 ¹
	Estriol (E3)	3.7*10 ⁻²
Synthetic	17 α -ethinylestradiol (EE2)	8*10 ⁻¹ - 3,3*10 ¹
	Nonylphenols	1.0*10 ⁻⁹ - 3.6*10 ⁻³
	Bisphenol A (BPA)	7.8*10 ⁻⁶ - 1.0*10 ⁻³
	Phthalates	1.1*10 ⁻⁵ - 3.2*10 ⁻⁸

THE AQUATIC COMMUNITY

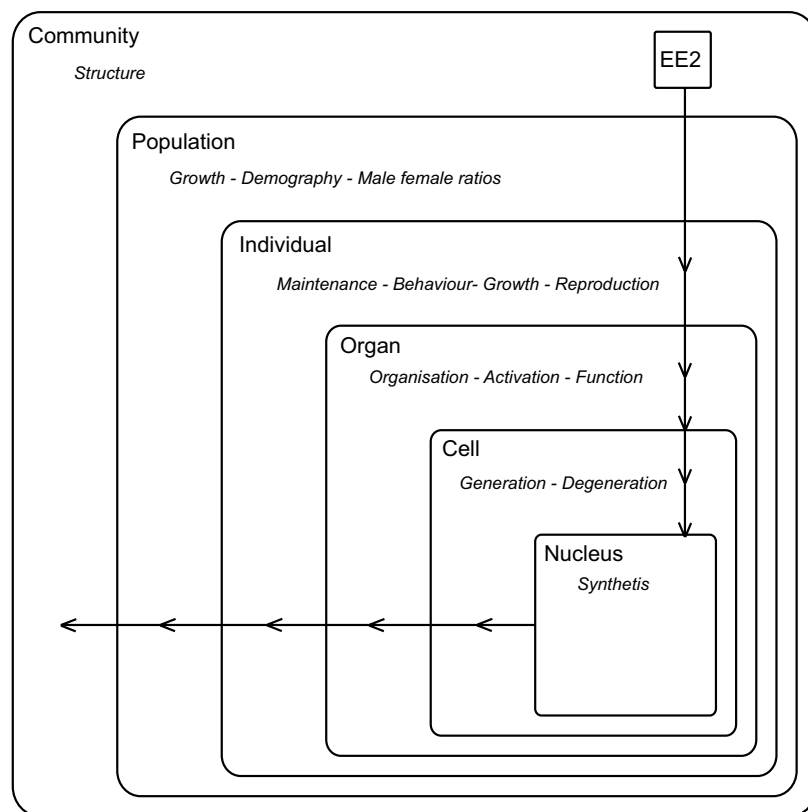


Figure 2. Simplified pathway (arrows pointing inwards) of endocrine disrupting compounds (EDCs) from emissions to surface waters, uptake in individuals, transport through tissues and binding to receptors on cell wall or inside nucleus. The following response (arrows pointing outwards) may be activation or deactivation of enzymes and/or proteins, cell generation or degeneration, size and/or functions of tissues or organs, changes in the individuals behavior or fertility, size and demography of populations and structure of the community, all depending on the how strong the effect was on the previous organizational level.

The aquatic community is composed of species that can be placed in different functional groups in the food web where they directly interact with the each other via predation, competition or mutualism that alter the behavior, or even the life status, for the individuals involved, as well as indirectly affect other species not directly involved. These direct and indirect interactions, as well as the species interaction with the abiotic environment, are the structuring forces that determine the size of populations and the species diversity in aquatic communities (Wootton 1994).

The abiotic environment can also come in the shape of anthropogenic stressors, such as chemical pollutants, and the interaction between the species and the chemical pollutant may be as strong as the biotic interactions and community ecology and animal behavior can be efficient tools to study and predict the effects anthropogenic chemicals may have in aquatic communities (Clotfelter et al. 2004; Rohr et al. 2006; Clements and Rohr 2009). For example, insecticides and herbicides designed to reduce the abundance of the target species have been shown to restructure aquatic communities by having both direct lethal and indirect non-lethal effects (Fleeger et al. 2003) as well as alter the behavior of the inhabitants (Steinberg et al. 1995; Saglio and Trijasse 1998).

The results from several studies show that the most sensitive species to estrogenic EDCs belong to the

vertebrate group (Caldwell et al. 2008; Segner et al. 2013) and this is not unlikely as EE2 is designed to mimic estradiol (E2) and modulate biological processes in humans. This can be related to the difference in how growth, sex differentiation, behavior, immune system and reproduction are controlled, either by in vertebrates or by neuropeptides in invertebrates (Köhler et al. 2007; Lafont and Mathieu 2007; Lagadic et al. 2007; Eick and Thornton 2011; Ahmed 2000; Pankhurst et al. 1999). It could be possible to divide aquatic species in two groups, with vertebrates on one side and invertebrates and plants on the other side, but this division should come with caution as there are receptors that have been conserved through evolution and can be found in both invertebrates and vertebrates (Gunnarsson et al. 2008).

With the difference in sensitivity to EE2 between fish and the majority of the other inhabitants in the aquatic community it is not unlikely that the negative effects on population size (Kidd et al. 2007) and sensory-motor systems (Nelson et al. 2008) in fish are indirectly affecting less sensitive prey species by reducing the predation pressure on them.

Up to the date when I started my PhD-student time there have been many studies performed on the effects of EE2, or other estrogenic EDCs, on different groups of species, for example, biotransformation and bioconcentration of EDCs in algae (Lai et al. 2002), crustaceans reproduction and mortality (Jukosky et al. 2008), larval molting

in insects (Watts et al. 2003) and fish physiology and reproductive behavior (Bjerselius et al. 2001). However, only a few studies have been performed on the effects on non-reproductive behaviors and higher organizational levels, for example fish anti predator behavior (Bell 2004), fish populations (Kidd et al. 2007) and plankton communities (Hense et al. 2004).

Therefore, the aim of this thesis was to investigate the effect EE2 may have on aspects of an organisms biology not necessarily connected to reproduction and the consequences on organizational level above the individual organisms, such as population growth rates and communities.

THE EFFECTS OF EE2 ON DIFFERENT ORGANIZATIONAL LEVELS, PAST AND PRESENT STUDIES

In **paper II** my colleagues and me exposed a pelagic community consisting of phytoplankton and zooplankton populations and later on roach larvae, mimicking hatching of planktivorous fish in temperate lakes, to EE2 at the nominal concentration of 50 ng/l. In this paper we used the approach of community ecology by viewing EE2 as an active predator (Rohr et al. 2006) that may inhibit fish foraging performance and indirectly affect species less sensitive to EE2. We showed that the effect of EE2 on the plankton community was low before adding the roach larvae. This result is in concert with several other studies showing that both phytoplankton and zooplankton do not seem to be sensitive to EE2 or other estrogenic EDCs.

However, there was a higher biomass of copepod nauplii stages in the control treatment suggesting more resources for the copepods in the control treatment or that reproduction was inhibited in the EE2 treatment. Effects of estrogenic EDCs on development or reproduction in copepods have previously only been seen at concentrations of 20 µg/L for both BPA and β-estradiol (Andersen et al. 1999) and 50 µg/L for EE2 (Andersen et al. 2001) and other have shown that only small (Jukosky et al. 2008) or absent effects (Breitholtz and Bengtsson 2001) even at concentrations of, or higher than, 50 µg/L for EE2 and 100 µ/L for BPA. Effects of EE2, or

other estrogenic EDCs, on possible food items for zooplankton, such as algae, have been shown on the energy flow and growth, but the concentrations of the EDCs (4-octylphenol, 4-nonylphenol, β-estradiol, bisphenol A (BPA) and EE2) where an effect was observed was at 200 µg/L for nonylphenol or higher for the other estrogenic EDCs (Perron and Juneau 2011; Liu et al. 2010). Algae can also promote biodegradation and biotransformation of EDCs (Liu et al. 2010; Della Greca et al. 2008) as well as bio-accumulate them (Lai et al. 2002; Correa-Reyes et al. 2007) with the possibility that they may be a source of EDCs to higher trophic levels (Cailleaud et al. 2011; Correa-Reyes et al. 2007). However, we did not determine the nauplii to specie and we could not say if their parents where grazers, predators or omnivors and therefore, the implications of our results with respect to nauplii remain inconclusive.

After releasing roach larvae to the mesocosm, the effects of EE2 were stronger than the period before. We showed that the biomass of copepods was higher and that the biomass of the roach was lower in the EE2 treatment. EE2 and other estrogenic EDCs have been shown to affect growth in fish, for example fathead minnow (*Pimephales promelas*) and zebra fish (*Danio rerio*) (Pawlowski et al. 2004; Van den Belt et al. 2002) and growth can even be regarded as sensitive

as reproductive end-points (Segner et al. 2013). EE2 can affect growth in fish by either modulating the activity of growth hormones shown in tilapia (*Oreochromis niloticus*) (Shved et al. 2008), increase the activity of detoxification (glutathione S-transferase (GST)) shown in Atlantic salmon (*Salmo salar*) (Greco et al. 2007) or increase the production of vitellogenin (vtg, a egg yolk precursor protein) shown in EE2 exposed Chinese rare minnow (*Gobiocypris rarus*) (Zha et al. 2008) where the two last processes likely to use energy that cannot be used for maintenance or growth.

That fish is sensitive to EE2 or other estrogenic EDCs have been shown in observations from surface waters receiving water from sewage treatment plants (STPs). Intersex (feminized testis) have been observed in gudgeon (*Gobio gobio*) (van Aerle et al. 2001) and in roach (Jobling et al. 1998), and increased vtg production have been found male rainbow trout (*Oncorhynchus mykiss*) (Harries et al. 1997; Harries et al. 1996). Experimental studies have found similar results with common carp (*Cyprinus carpio*) (Gimeno et al. 1998) and roach (Lange et al. 2008). Hence, the effects of EE2 are strongest at the top of the food web and seem to diminish further down.

This suggests that our observed effect on roach weight was a directly consequence of EE2 exposure and that the zooplankton biomass was indirectly affected. In order to investigate why the biomass of copepods was higher and roach biomass was lower we exposed roach, from eggs to 84 days post hatch, to 50 ng/L EE2 in a second experiment. At day 84 we tested if the foraging

performance was inhibited in the EE2 exposed roach by measuring the amount of *Daphnia magna* that was consumed. Here we observed that the EE2-exposed roach consumed approximately 20 percent of the *D. magna* provided while the unexposed roach consumed approximately 80 percent, i.e. EE2 had a dramatic effect on foraging success. This reduction in foraging performance may explain the higher biomass of zooplankton and lower biomass of roach in the EE2 treatment of mesocosm experiment.

Estradiol, and other hormones, is involved in several processes in fish, e.g. reproductive behaviors (Pankhurst et al. 1999), therefore are there several studies showing that male behavior is modulated by EE2. Inhibition in aggressiveness towards competing males (Bell 2001) and a change in social hierarchies among males (Colman et al. 2009) are just a few examples showing that EE2 have effects on such behaviors. The roach used in the foraging experiment were juveniles and therefore their behaviors were not connected to reproduction (see box 1 regarding the effects of estrogenic EDCs on fish reproductive behavior and fertility).

Hence, the change in foraging performance suggests that EE2 may affect the sensory-motor system and thus affecting the locomotor activity making fish less active (Nelson et al. 2008). EE2 have been found to affect locomotor activity in fish exposed from an early life stage, e.g. pipefish (*Syngnathus abaster*) migration from bottom to surface (Sarria et al. 2011a), swimming burst (Sarria et al. 2011b) and swimming activity (Reyhalian et al. 2011) in zebra

Box 1. The effects of EE2 or other estrogenic EDCs on reproductive behavior and fertility in fish.

Fertility and reproductive behavior in female fish is under control of 17 β -estradiol (E2) (Pankhurst et al. 1999) and these activities may be triggered by the environment such as the temperature (Goulet 1995). As EE2 mimics E2 there are several studies showing that especially male fish, in several species, are sensitive to EE2. For example, EE2 have shown to reduce their aggressiveness toward competing males (Saaristo et al. 2010b), to inhibit their parental care (Saaristo et al. 2010a) as well as change reproductive hierarchies and sexual selection (Coe et al. 2008) and these are just a few studies exemplifying these effects. Reduction in fertility has also been seen in males where EE2 caused by non-functional testes (Nash et al. 2004) or reduction in number of sperms (Kristensen et al. 2005) but also development in eggs have been shown to be inhibited (Kime and Nash 1999). No wonder that EE2 exposed fish populations may be driven to extinction (Kidd et al. 2007).

fish (*Danio rerio*). Organizational deformities such as abnormal development of the vertebrate column have been shown in mummichogs (*Fundulus heteroclitus*) exposed to 17 β -estradiol (E2) (Urushitani et al. 2002) and in fathead minnow exposed to EE2 (*Pimephales promelas*) (Warner and Jenkins 2007), and this may also affect their locomotor activity.

In **paper II** we exposed roach to EE2, from fertilized egg to their juvenile stage and found that their foraging performance was inhibited but we could not assess where in the foraging cycle EE2 may affect fish and if EE2 would have the same affect the foraging performance in adult fish and this initiated both **paper III** and **IV**. Therefore, in **Paper III**, I exposed adult crucian carps (*Carassius carassius*), a cyprinid just as roach, to 0, 1, 10 or 100 ng/L EE2 for 21 days and thereafter

quantified different activities during foraging such as swimming speed, distance swum and capture success when foraging on *Gammarus pulex*, as well as the physiological health such as length, weight, condition factor and size of liver and gonads. In **paper IV** I also exposed adult crucian carps (*Carassius carassius*) but to 0, 10 or 100 ng/L EE2 for 56 days and here I used a mesocosm experiment, with a prey community (zooplankton and macroinvertebrates) and algae community (periphyton and phytoplankton), with the same community ecology approach as **paper II**. In **paper IV** I also quantified length, weight, condition factor, size of liver and gonads in the crucian carps as well as the biomass of the prey community and algae community (chl- α).

In the crucian carp foraging experiment I did not observe any effects of EE2 on any of the quantified foraging

activities and the difference in effects on foraging performance compared to the EE2 exposed roach in **paper II** may be related to organizational effects in the developing roach as we explained earlier. The EE2 exposed adult crucian carps are not likely to exhibit any organizational deformities but activational changes may occur, such as the size and functions of liver or gonads, and an increase in liver size was seen in both male and female crucian carps and the reduction in gonads was seen in the males, both in the foraging and mesocom experiment.

Other studies have also observed effects of EE2 or other estrogenic EDCs on the size of liver, for both males and females, in three-spined sticklebacks (*Gasterosteus aculeatus*) (Andersson et al. 2007) and rare minnows (*Gobiocypris rarus*) (Zha et al. 2007). Effects of EE2 have also been observed in gonads where the effect where more severe in males than females as seen in fathead minnows (*Pimephales promelas*) (Pawlowski et al. 2004). The increase of liver size is likely due to an increased vtg production as this synthesized in the liver (Andersson et al. 2007) and the decrease in gonad size in the males was probably due to degeneration of germ cells (Miles-Richardson et al. 1999). Studies have also shown that fish exposed to EE2 have reduced glycogen storage (Olivereau and Olivereau 1979; Ekman et al. 2008) and a reduction in energy storage may have implications during seasons where food may be scarce.

I also showed that EE2 affected crucian carp weight and condition factor in the foraging experiment and weight, length and condition factor the mesocosm experiment, i.e. largely

similar results as for the EE2 exposed roach in **paper II**. However, the effects were more severe in the crucian carps in the mesocosm experiment as length was also affected. This is not unlikely as the EE2 exposure period was 56 day compared to 21 days in the foraging experiment. Also, the condition factor was reduced in the highest treatment (100 ng/L EE2) in both of the crucian carp experiments, suggesting a large effect induce by EE2.

The inhibited growth in the EE2 exposed roach in **paper II** was explained by either an inhibition of foraging performance, modulation of growth hormones or processes that requires energy, such as increased vitellogenin production and detoxification. There were however no significant effects on the foraging activity in the EE2 exposed adult crucian carps, and only weak effects on the prey community. Therefore, it is not likely that reduced food intake caused the reduced growth in the crucian carps. I observed during the exposure period before the foraging activity trials that more food uneaten in the highest treatment of 100 ng/L EE2 compared to the other three treatments and also a weak, but not significant, trend that the foraging success was lowered by EE2 exposure.

The sensitivity to EE2 or other estrogenic EDCs varies in prey community (*Asellus aquaticus*, *Gammarus pulex*, *Radix balthica*, *Bithyina tentaculata* and zooplankton) for the crucian carps in the mesocosm experiment. Amphipods, such as *Gammarus pulex* have been found to respond to EE2 and Watts et al.(2002) showed that population size was larger

in treatments of 1 µ/L and higher and also that there were more females in these treatments, similar results shown by Vandenberg et al. (2003). To my knowledge here are no studies performed on the effects of EE2 or other estrogenic EDCs on *Asellus aquaticus* and they used as indicator species in polluted waters (Whitehurst 1991) but their land living relative, *Porcellio scaber*, have been shown have reduced reproductive output (Lemos et al. 2010).

However, gastropods have been found sensitive to EE2 and other estrogenic EDCs and studies have shown increased reproduction in *Potamopyrgus antipodarum* exposed to EE2 or BPA at concentrations of 25 ng/L and 5 µg/L, respectively, and that somatic growth can either be enhanced for *R. balthica* or inhibited for *B. tentaculata* at 5 ng/L EE2 (**Paper I**). Even if the gastropods used in the crucian carp mesocosm experiment are sensitive to EE2 there is nothing that suggest that EE2 may have increased the reproduction and thereby enhanced the growth of the crucian carps (**Paper 1**).

Gastropods have been proposed as model organisms in studies of EDC effects (Matthiessen 2008) and there are a few studies done showing that they are sensitive to EE2 or other estrogenic EDCs. In **paper I** my colleagues and me exposed two different snail species to EE2, from 0.5 ng/l up to 50 000 ng/l, in a concentration-response experiment, covering levels of EE2 that can be found in surface waters up. The species were *Radix balthica*, a hermaphrodite, and *Bithynia tentaculata*, a species with separate sexes. Hence, the two species have very different reproductive strategies. In this paper we quantified

different life history traits (mortality, somatic growth, time to reproduction, size at reproduction, amount of eggs and hatching success) and calculated their population growth rate. There were no effects of EE2 on any of the variables used for population growth rate and therefore no effect was seen on population growth rate but there was an effect on somatic growth rate, enhanced for *R. balthica* and inhibited for *B. tentaculata*.

If the difference in somatic growth rate is related to their different reproductive strategies is however not known. Mollusks have been quite extensively used because they may respond to low concentrations of EE2 or other estrogenic EDCs but the response may depend on the life-history stage, species or exposure concentration (Jobling et al. 2003; Benstead et al. 2011; Andrew et al. 2010). Androgen-estrogen metabolizing enzymes (CYP450 arotmases) and genes coding for estrogen receptors orthologs have been shown in mollusks (Koehler et al. 2007; Wootton et al. 1995; Hultin et al.) but the role of these estrogen receptors and if EE2 have any endocrinological role is however unknown (Scott 2013).

Furthermore, there are not only estrogenic EDCs in the aquatic environment; there are many other anthropogenic chemicals with different mode of action. Factors such as exposure history can also make organisms more or less sensitive to a chemical which may come with a cost (Wilson 1988), and also affect how an organisms may respond to a novel stressor (Clements and Rohr 2009). Mollusks, with their sedentary life style, global distribution

and sensitivity to EE2 can make them good contenders to study the effects how pollution history may alter the effects of novel chemicals. In **paper I** we showed that somatic growth rate was enhanced in *R. balthica* when exposed to EE2 but this does not seem to come with a cost later in life as there were no negative effects on the reproductive output.

In **paper V**, my colleagues and me exposed two different populations of *R. balthica* to EE2 and cadmium, two chemicals with different mode of action. One population was born and reared in an EE2 environment (second generation from **paper I**) and one population came from non-EE2 exposed population in a pond. In this paper we used somatic growth to evaluate the effect of EE2 exposure history and the effect of a novel stressor, cadmium. As we used two different populations with different life history, except previous EE2 exposure, we analyzed growth rate as percent and relative growth.

In one experiment we exposed the pond population to cadmium (0, 50 and 200 μL) and we did not observe any effect on the somatic growth rate and this insensitivity may be related to that they have metallothioneins to bind to metals and detoxify them (Amiard et al. 2006). In a second experiment we exposed both populations to 50 ng/L EE2 without or with cadmium (0, 50 and 200 μL) and here we showed that the EE2 population had a higher growth rate (percent) compared to the pond population but there was no difference in relative growth rate. This may suggest that the EE2 population may be less sensitive to EE2 but we cannot disregard the different life histories. Both populations were

however sensitive to the simultaneous exposure of cadmium and EE2 and this suggest that cadmium induces a larger burden and this is not unlikely as they have different mode of action. It is not clearly known how EE2 acts in mollusks (Ketata et al. 2008) but cadmium is metal that may induce oxidative stress (Stohs and Bagchi 1995) and detoxification processes are also likely to be different, metallothioneins for detoxification and excretion of metals (Amiard et al. 2006) and EE2 may be metabolized by GST (Baturu and Lagadic 1996).

The activity of GST have been found to be decreased in EE2 exposed copepods (**Paper VI**) that can lower the protection against other chemicals, including metals (Lee et al. 2007). There were no effect of cadmium on the pond population in the first experiment and this may suggest the inhibited growth in both populations was induced by the simultaneous exposure of EE2 and cadmium. There are studies showing that invertebrates can be tolerant to metals and pesticides such as gastropods (Lefcort et al. 2004), earthworms *Dendrobaena octaedra* (Rozen 2006) and insects (Raymond et al. 2001). There are however few studies showing if organism can become more tolerant to EE2, or other estrogenic EDCs, but killifish (*Fundulus heteroclitus*) have been observed to be more tolerant to E2 after living for generations in waters polluted with polychlorinated biphenyls (PCBs) (Greytak et al. 2010).

Earlier in **paper II** and **IV** we used zooplankton, including copepods, as prey for both roach and cichlid carps. In **paper VI** my colleagues and me exposed copepod populations to 0, 10,

100 and 1000 ng/L EE2 and quantified physiological effects that may affect the locomotor activity, development and the ability to metabolize xenobiotic chemicals. We showed that EE2 decreased the activity Glutathione S-transferase (GST) (detoxification) and Caspase-3 (CASP-3) (apoptosis), for both calanoida and cyclopoida copepods, which can make them more susceptible to novel chemical stressors (Lee et al. 2007) and delay molting during their nauplii and copepodite stages and also the metamorphosis from nauplii to copepodites (Andersen et al. 2001). Furthermore, we also showed that the activity of acetylcholinesterase (AChE) (neurotransmission) decreased for cyclopoids but not for calanoids and that EE2 affected the ratio between adult calanoids and copepodites. However, total abundance, both adults and copepodites, was not affected.

In the roach mesocosm experiment (**Paper II**) where we exposed juvenile roach and a plankton community to EE2, we observed that the nauplii biomass was higher in the control treatment before we added the roach, but we could not come up with any reasonable explanation to this. It is not unlikely the decreased activity of CASP-3 may have delayed the development from copepodites to adults and thereby delaying time to reproduction. I therefore analyzed ratio between copepodites and adults, for both cyclopoids and calanoids, in the roach mesocosm experiment but there were no effect of EE2, before or after adding the larval roach (Figure 3). However, the copepodite to adult ratio for cyclopoids was higher than for calanoids after that that the roach was added.

In **paper II** we explained the increase in copepod biomass by their anti-predator behavior, compared to the more listless cladocera, but the effects of EE2 seen in the activity of AChE suggest that cyclopoids may be more vulnerable to predation compared to calanoids. However, we could not determine if cyclopoids were more vulnerable, as the pattern of abundance or biomass was similar to those of calanoids. The physiological endpoints here, GST and CASP-3, were affected already at 10 ng/L EE2 and that is effects at lower concentrations than many other studies (Andersen et al. 2001; Jukosky et al. 2008; Hutchinson et al. 1999) even though copepods lack orthologue receptors of EE2 (Breitholtz and Bengtsson 2001) and this warrants more research.

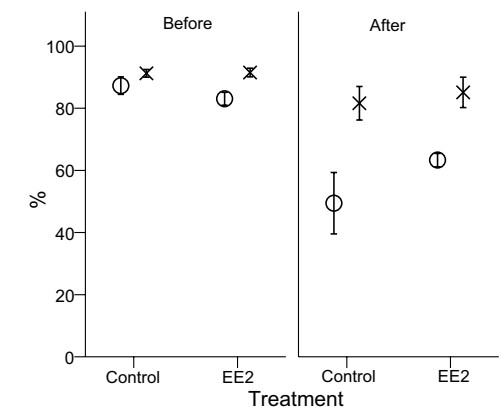


Figure 3. Ratio between copepodites and adults for calanoida (O) and cyclopoida (X) copepods in control and EE2 treatments before and after roach was added to the experimental arenas (Paper II).

CONCLUSION

Is there anything more to do in the research around EE2, or other EDCs, and the eventual consequences for the inhabitants of the aquatic sphere? This could have been asked in the beginning of my PhD-student days and Sumpter and Jobling (2013) wrote “Do we need to know more” implying that the most important question regarding the effects of synthetic estrogens are addressed and answered and that research should be directed elsewhere. This paper was published at the end of my PhD-student days and I must say that I do agree with them, at least to some extent. When looking at the results in the papers that me and my colleagues have produced, the effects of EE2 on the different organizational levels does not seem to be strong, at least in the light of the amount of EE2 found in surface waters.

However, there are indications that more severe effects on the physiological health are observed with longer EE2 exposure, at least for species sensitive to EE2 and maybe other estrogenic EDCs. When taken all results together, our and others studies, effects such as reduced physiological health, infertility, inhibited foraging performance, inhibited reproductive behavior in fish and the low, or no, effects of EE2 on the majority many other inhabitants in the aquatic community there seems to be winners (invertebrates) and losers (vertebrates) with possible changes in community structures and ecosystem functions that warrant attention.

With this in mind, we actually know very little about the effects that EE2 or other estrogenic EDCs may have in the natural aquatic communities, probably because the isolated effects they may have are difficult to observe in the natural environment as they are vast in both space and time (Kohler and Triebkorn 2013), so there is much more to do as we only have scratched the (water)surface of these communities. There are also other aspects that are not much studied together with the effects of EE2 or other estrogenic EDCs such as chemical mixtures, organisms exposure history, the added stress from biotic interactions and other non-chemical abiotic stressors as well as and the effects during the ontogeny, and not only in fish.

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Thanks

This has not been an easy road to follow, full of bumps and potholes, and sometimes taking the wrong turn, or just going straight out into the shrubs, hoping for a tow truck to show up, but now, sitting here and typing the final words it seems that I have reached the destination. I am not a person that uses too many words so the meaning of my thanks and gratitude, to all the people that I have met and worked with, are greater than the amount of words that I have used to express it.

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MY CONTRIBUTION TO THE PAPERS

The idea and design for paper I was developed by Anders Persson (AP), Olof Berglund (OB) and me with additional information about *R. balthica* and *B. tentaculata* from Thomas Lakowitz. I performed the experiment together with AP and Saioa Zorita (SZ) did the analysis of EE2. I analyzed the data and I wrote the paper with contribution from AP, OB and SZ.

The set-up for the mesocosm experiment in paper II was provided by Lars-Anders Hansson (LAH) and AP initiated the idea of our participation in the project. LAH, AP, Alice Nicolle (AN), Christer Brönmark (CB), Emma Kritzberg (EK), Villem Granelli, Jessica von Einem (JvE) and I participated during the initiation of the project. Sampling and analysis was performed by LAH, AN, AP, EK, CB, JvE and me and analysis of EE2 was done by Murtasa Hyder (MH). The idea and design of the foraging performance experiment was mine and Lina Nikoleris and I performed the experiment. Analysis of data was performed by me and I wrote the paper with contribution from AP, LAH and CB.

I developed the idea and design for paper III together with AP. I performed the experiment, analyzed the data and wrote the paper with contribution from AP.

I developed the idea and design for paper IV with participation from AP. I analyzed the data and wrote the paper with participation from AP.

AP and OB developed the idea and design for paper V and Alexandra Nilsson (AN) performed the experiment with participation from me. I analyzed the data and wrote the paper with participation from AP, OB and AN.

Maria Sol Souza (MSS) provided the idea and design for paper VI with participation from LAH, The experiment was performed by MSS with participation from LAH and me. MSS analyzed the data and wrote the paper with participation from LAH, Esteban Balsario and me.

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