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Parent-to-offspring transfer of sublethal effects of copper exposure: Metabolic rate and life-history traits of *Daphnia*

Transferencia paterno-filial de efectos subletales de la exposición al cobre: Tasa metabólica y rasgos de historia de vida en *Daphnia*

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ABSTRACT

In ecological communities, pollution driven perturbations exert immediate effects on sensitive individuals, but these effects may be transmitted among interacting organisms and spread over the community through several paths. This makes the assessment and prediction of ecological consequences of pollution difficult. The propagation of perturbation effects among organisms can be horizontal among organisms that coexist in space and time, and vertical among organism that belong to different generations. The latter process is poorly understood, in particular in planktonic organisms facing metal pollution. In this study we evaluate the vertical transfer of effects driven by sublethal copper stress on the heartbeat rate, somatic growth and fertility of *Daphnia pulex*. In order to evaluate this, we performed a factorial experiment in which parental and filial generations were exposed to both copper-enriched and control media. We found that parental exposure to copper exerted a significant effect on the heartbeat rate, somatic growth and fertility of offspring, revealing a transgenerational effect in *D. pulex*. This response may be explained by a higher resource investment on repair/detoxification processes in the parental generation, allocating fewer resources to offspring quality. Our results suggest that responsiveness of organisms to stress is dependent on parental history.

Key words: ecotoxicology, heartbeat rate, maternal effects, transgenerational effects, zooplankton.

RESUMEN

En las comunidades ecológicas, las perturbaciones producidas por los contaminantes ejercen efectos inmediatos en los individuos sensibles, pero estos efectos podrían ser transmitidos entre los organismos interactuantes y extenderse sobre la comunidad a través de múltiples vías. Esto hace difícil la evaluación y predicción de las consecuencias ecológicas de la contaminación. La propagación de los efectos de una perturbación entre los organismos puede ser horizontal, entre organismos que coexisten espacial y temporalmente, y vertical, entre organismos que pertenecen a generaciones diferentes. Este último proceso ha sido escasamente entendido, en particular en organismos planctónicos enfrentados a contaminación por metales. En este estudio evaluamos la transferencia vertical de los efectos producidos por un estrés subletal de cobre sobre la tasa de latidos cardiacos, el crecimiento corporal y la fertilidad de *Daphnia pulex*. Para evaluar esto, se realizó un experimento factorial en el cual las generaciones parental y filial fueron expuestas tanto a medios enriquecidos con cobre como a medios control. Encontramos que la exposición de las madres al cobre ejerció un efecto significativo en la tasa de latidos cardiacos, el crecimiento corporal y la fertilidad de la descendencia, revelando un efecto transgeneracional en *D. pulex*. Esta respuesta puede explicarse por una mayor inversión de recursos en los procesos de reparación/desintoxicación en la generación parental, asignando menos recursos a la calidad de su descendencia. Nuestros resultados sugieren que la reactividad de los organismos al estrés depende de la historia parental.

Palabras clave: ecotoxicología, efectos maternos, efectos transgeneracionales, tasa de latidos cardiacos, zooplancton.

INTRODUCTION

The study of effects of metal pollution on aquatic biota has attracted significant attention in the last decades since many industrial processes have contributed to this source of disruption in freshwaters (Dolédec & Statzner 2010), a resource that is being depleted at an accelerating rate worldwide. Metal exposure is known to exert a diverse array of harmful effects on aquatic organisms. In invertebrates, examples include oxidative stress (Barata et al.

2005), elevated oxygen consumption (Gerhardt 1995), lowered feeding rate (Blockwell et al. 1998), altered locomotion (Untersteiner et al. 2003) and decreased individual growth rate (Winner 1985). Nevertheless, understanding and predicting the ecological consequences of anthropogenic disturbances and pollutants in particular on natural ecosystems is difficult, since the immediate effects of perturbations on sensitive individuals may be transmitted among interacting organisms and spread through the community via multiple pathways.

Borrowing the concept from genetics, two sources of ecological transfer of effects among organisms can be distinguished. Horizontal transfer of effects takes place among organisms that coexist in a given time and place and whose biological performance depends on each other. For example, an herbicide could impair reproduction of plants but indirectly lead to decreasing abundances of herbivores whose resources were depleted (e.g., Lampert et al. 1989, Juttner et al. 1995, Kasai & Hanazato 1995), and ultimately decrease the abundance of carnivore predators that depend on herbivores (Rhor et al. 2006). Horizontal transfer leads to what is customarily known as indirect effects (Wootton 1994). Vertical transfer, on the other hand, refers to propagation of effects within an ecosystem among organism that belong to different generations. For example, organisms could express a response to a contaminant to which only their parents were exposed. This kind of phenomenon is usually known as maternal effects (Bernardo 1996), transgenerational effects (Marshall 2008), latency (Brock et al. 2008) or delayed effects (Beckerman et al. 2002). Both horizontal and vertical transfer of effects among organisms constitute two main sources of ecological complexity whose insufficient comprehension has hampered our advancement in the assessment of environmental risk associated to actual or potential human-driven perturbations.

Although there is an increasing interest of scientists in studying horizontal transfer of pollutant effects in ecological communities (Clements & Rohr 2009), less attention has been paid to the study of vertical transfer, which has been evaluated in only a few taxa and mainly considering their consequences on organisms' life-histories (Hammers-Wirtz & Ratte 2000, Lin et al. 2000, Marshall 2008, Kwok et al. 2009). However, although life-history traits are related directly to fitness, they are prone to be constrained by trade-offs among them. Therefore, characterizing life history and physiological parameters allows one to better capture the range of potential effects of perturbations. Consequently, in this study we evaluate the vertical transfer of effects driven by sublethal copper stress on the metabolic rate (measured as heartbeat rate), somatic growth and fertility of *Daphnia pulex* (Leydig 1860). This herbivore species is a cosmopolitan inhabitant of ponds and lakes and constitutes an important link in aquatic food webs, transferring energy from producers towards large predatory species. In addition, daphnids are efficient controllers of algal biomass and bacterial growth. Hence, the biological activity of *Daphnia* exerts a strong influence on community dynamics, lake metabolism and ultimately the generation of ecosystem services (Edmonson & Litt 1982; Lampert et al. 1986). In copper-exposed daphnids, metabolic rate (and hence heartbeat rate) is expected to increase due to a higher energetic demand driven by induced biochemical and physiological mechanisms of resistance. As a consequence of a higher allocation of resources to maintenance mechanisms, growth, development and reproduction rates are expected to be lowered (Jager et al. 2006). If vertical transfer does not take place, these effects are expected to be independent of the parental environment.

METHODS

Daphnia cultures

Stock cultures of *Daphnia pulex* were obtained from Bagshaw pond, England (53°20'5.37" N, 1°27'8.12" W). We used this strain because we had several English clones genotypified for future comparative analyses. Nevertheless, after this study all individuals coming from cultures of English strains were destroyed. Twenty females were kept in the laboratory in individual 50 mL vials with 40 mL of hard water (ASTM 1980), under 14:10 D:L photoperiod, temperature of 20 ± 2 °C, and pH 7.8 ± 0.2. The cladocerans were fed with *Chlorella vulgaris* at a density of 10^6 cells mL^{-1}.

Prior to conducting the experiment, we determined the sensitivity of *D. pulex* to copper using an acute bioassay to estimate the LC50 (modified from EPA 1993). The nominal copper concentrations used to perform the bioassay were 25, 50, 75, 100 and

125 µg L^{-1} Cu^{+2}. The test medium was supplemented with *Chlorella vulgaris* 10^6 cells mL^{-1} and enriched with 2.5 mL L^{-1} of nutritional supplement Phyllum (Hayashi et al. 2008) in order to replicate the experimental conditions to be used later.

Experimental setup

Twenty neonates (< 24 h) were placed individually into 50 mL vials with 40 mL of medium without copper (control medium). From their third brood we took 40 neonates. Twenty of them were placed individually into 50 mL vials with 40 mL of control medium. The remaining 20 neonates were placed individually into 50 mL vials containing 40 mL of 20 µg L^{-1} Cu^{+2}. All media were renewed every 24 hours, until day 12 when animals released their third clutch. These animals constitute the parental generation F0.

From the third brood of each of the parental groups (F0), we took 50 randomly chosen neonates (F1). Twenty-five of the F1 daughters coming from the parental control group (C group) were placed individually into vials with 40 mL of control medium (C-C group). The other 25 coming from the parental control group were individually exposed to 20 µg L^{-1} Cu^{+2} (C-Cu group). Experimental media were renewed every 24 hours, until the third day. The same procedure was performed with the F1 individuals whose mothers were exposed to copper, obtaining in this way the treatments Cu-C and Cu-Cu. This design corresponds to a balanced 2 x 2 full factorial design with parental environment (with and without copper) and filial environment (with and without copper) as experimental factors.

Size-specific heartbeat rate

We estimated the heartbeat rate (HB min^{-1}) of *D. pulex*, corrected by individual body mass in mg. For calculating body mass, measured body length was transformed to mass following a standard empirical regression for *Daphnia* (Bottrell et al. 1976). For measuring the heartbeat rate we impeded locomotion of the experimental organisms by means of the following treatment. We first placed each individual on a slide using a Pasteur pipette. Then the surrounding water was removed. Using a dissecting needle, each individual was attached by its helmet to a line of vaseline disposed on the slide and medium was added immediately. This step took less than 10 sec. By this procedure, the abdomen kept free thus allowing easy access of water to the gills. Five individuals were disposed on each slide, separated 1 cm from each other. Finally, the slide was placed in a petri dish filled with 70 mL of hard water (ASTM 1980), at a temperature of 20 ± 2 °C, pH 7.8 ± 0.2 and constant diffuse light. Measurements of individuals' heartbeat rate were made at the third day from birth, on digital videos (3 x 10 seconds) recorded with a digital camera attached to a microscope. For assessing the acclimation time needed prior to the measurements, we observed the variation of heartbeat rate of single individuals through reading that parameter every 30 minutes, over a period of 14 hours from the mounting procedure.

Life-history traits

We examined shifts in somatic growth and fertility caused by copper exposure. For analyzing somatic growth we measured the body length of the F1 individuals at the beginning of the experiment (neonates) and then at the end of experiment (3-day old). Length measurements were made using digital images obtained from a digital camera attached in a microscope focused (top down) on the slide, keeping individuals at the same focal point. The precision of the length measurements was 10^{-4} mm. For fertility data, we inspected all individuals every day and annotated the clutch size at first reproduction.

Statistics

Data were analyzed by means of two-way randomization ANOVA (1500 randomizations over the original values), for testing the effects of parental and filial environments and their interaction on size-specific heartbeat rate, somatic growth and fertility. All analyses were done with the package RT2.1. For technical details about the randomization procedures the interested reader could see the accompanying text (Manly 1997).

RESULTS

Copper tolerance and acclimation time

The acute bioassay resulted in an LC50 value of 60 µg L^{-1}. This means that 24 h copper exposure at a concentration of 60 µg L^{-1} caused mortality of 50 % of experimental *Daphnia*. Following Koivisto et al. (1995) we decided to use 1/3 of the LC50 value (20 µg L^{-1}) for all subsequent experiments.

The heartbeat rate of a few tested individuals stabilized after 10 hours from the mounting procedure. Based on this result, we set the acclimation time for all measurements of heartbeat rate to be 12 hours.

Size-specific heartbeat rate

Both parental and filial environments, as well as their interaction, exerted a significant effect (Table 1) on the heartbeat rate of *Daphnia*. The observed differences were mainly driven by the high heartbeat rate resulting from the Cu-Cu treatment (Fig. 1A).

Life history traits

Neonates of F1 with different parental environments did not differ significantly in their body lengths. Therefore, final body length became a direct measurement of somatic growth. Both environments as well as their interaction exerted a significant effect on final body length (Table 1). The differences in body sizes were mainly due to a reduced body length of organisms in the Cu-Cu treatment (Fig. 1B).

Clutch size (Fig. 1C) also responded significantly to both parental and filial environments, but their interaction was not significant (Table 1). This means that copper exposure decreased fertility of organisms that were directly exposed to the stressor and that of organisms whose mother had been exposed, in an additive way (Table 1). Throughout our experiments, no mortality occurred in any treatment.

DISCUSSION

Our results revealed a transgenerational effect of copper exposure across generations in *Daphnia*. In particular, we observed that individuals whose mothers were exposed to copper showed a significant increase in heartbeat rate and a decrease of somatic growth rate when they also developed under a polluted environment, as compared to individuals whose mothers were not exposed to the pollutant. Similarly, fertility was decreased by copper exposure in F0 and F1, but the interaction of both environments was not significant.

The transgenerational responses observed in our experiments may be explained by a higher resource investment in detoxification processes in the parental generation, allocating fewer resources to offspring quality (see Sibly 1996, Kooijman et al. 2009). Consequently, exposed daughters had a higher expenditure on repair/detoxification processes and poorer energy allocation to somatic and reproductive investment. Increased energy demand by detoxification/repair processes may be attributable to the synthesis of defense proteins (Cousins 1985, Smith et al. 2001, Kil et al. 2006, Rose et al. 2006, Wu et al. 2006), and specifically, increased synthesis of metallothionein, which is considered to be a highly energy-demanding process under sublethal metal exposure in daphniids (Barber et al. 1990). Also, copper could produce lipid peroxidation, which has been shown to increase the levels of glutathione peroxidase in *Daphnia* (Barata et al. 2005). Consequently, the observed decrease in somatic growth and reproduction is

TABLE 1

Summary of two-way randomized ANOVA for the three response variables: fertility, body growth and heartbeat rate. The two factors were the environment (with or without copper) of the parental (F0) and filial (F1) generations.

Resumen de ANDEVA de dos vías por aleatorizaciones para las tres variables respuesta: fertilidad, crecimiento corporal y tasa de latidos cardiacos. Los dos factores fueron el ambiente (con y sin cobre) de la generación parental (F0) y filial (F1).

	DF	SS	MS	F	P
Fertility					
F0 environment	1	210.20	210.20	26.2	< 0.0001
F1 environment	1	106.10	106.10	13.13	0.0005
Interaction	1	16.81	16.81	2.08	0.1525
Error	96	775.60	8.08		
Body growth					
F0 environment	1	0.89	0.89	22.86	< 0.0001
F1 environment	1	0.43	0.43	10.96	0.0014
Interaction	1	0.37	0.37	9.53	0.0027
Error	88	3.44	0.04		
Size-specific heartbeat rate					
F0 environment	1	451.00	451.00	19.5	< 0.0001
F1 environment	1	503.30	503.30	21.76	< 0.0001
Interaction	1	430.70	430.70	18.62	< 0.0001
Error	52	1203.00	23.13		

consistent with higher resource allocation to maintenance (Baillieul et al. 2005, Zeman et al. 2008, Massarin et al. 2010). Elevation of metabolic rate, measured as oxygen consumption, driven by exposure to metal ions is a common phenomenon, which has been reported for both invertebrates (Thurberg et al. 1974, Knop et al. 2001, Vosloo et al. 2002) and vertebrates (Rowe et al. 1998, Hopkins et al. 1999, Tatara et al. 2001). An alternative hypothesis that is consistent with our experimental findings is that Cu itself could be transferred to offspring (Zhao et al. 2009), causing some degree of physiological stress on daughters. This may account for the slight reductions in the offspring that were not subjected to the second round of Cu exposure, and the significant reductions in the offspring that were subjected to Cu. In this case, vertical transfer of effects could be caused by vertical transfer of the metal itself, an hypothesis considered by Lin et al. (2000).

Transgenerational pollutant effects have been reported for wide range of taxa (Lin et al. 2000, Hopkins et al. 2006, Marshall 2008, Ho & Burggren 2010). In *Daphnia*, previous work presented cases where vertical transfer of adverse effects led to reduced offspring fitness by affecting some life-history traits. This has been reported for metal pollutants such as cadmium (Guan & Wang 2006) and uranium (Massarin et al. 2010), as well as for other stressors such as UV radiation (Huebner et al. 2009).

It has become clear that parent-to-offspring transfer of effects is not rare in nature (Beckerman et al. 2002), but its consequences on population and community dynamics remains a topic requiring further advances. With regard to vertical transfer of effects driven by pollutant exposure, our results indicate that responsiveness to stress of organisms' traits is strongly dependent on parental history. Therefore, the appropriate assessment of ecological risk associated with toxic substances released to the environment by industrial activities could be seriously flawed if they were based on immediate effects only. More realistic toxicity tests performed on successive generations could provide valuable information about the actual threat associated to a given stressor on wild species, and constitute a more sensitive probe for

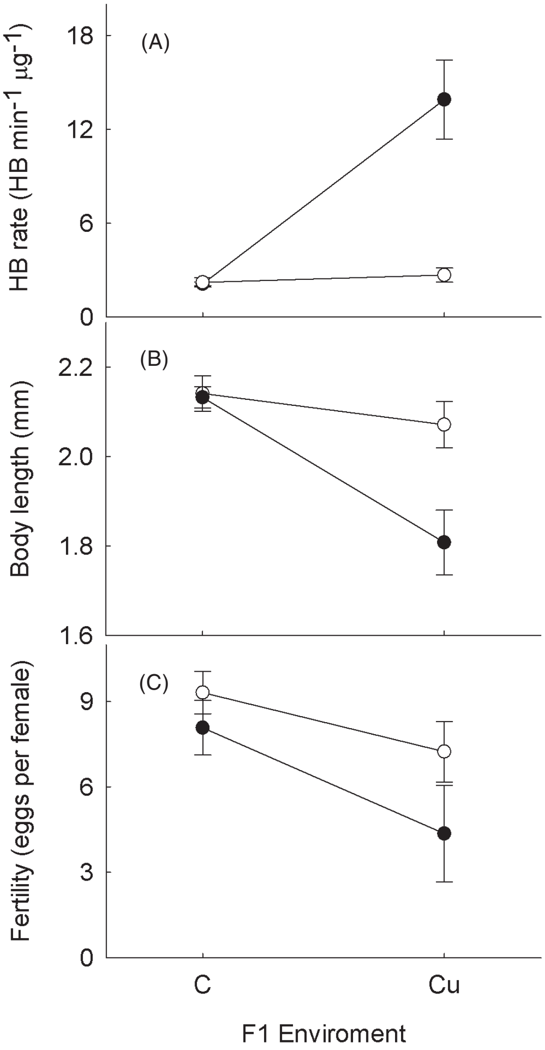

Fig. 1: Size-specific heartbeat rate (A), juvenile body length (B) and fertility (C) of *Daphnia pulex* as a function of parental and filial exposure to copper. Means ± 95 % confidence intervals are shown. Closed and open circles show results for organisms whose mothers (F0) were exposed and not exposed to copper, respectively. Filial (F1) environment is displayed on the x-axis. C: control medium, Cu: copper-added medium.

Tasa tamaño-específica de latidos cardiacos (A), longitud corporal juvenil (B) y fertilidad (C) de *Daphnia pulex* en función de la exposición parental y filial al cobre. Se muestran valores promedio ± intervalos de confianza de 95 %. Círculos negros y blancos muestran resultados para organismos cuyas madres (F0) fueron expuestas y no expuestas al cobre, respectivamente. El ambiente filial (F1) se muestra sobre la abscisa. C: medio control, Cu: medio con adición de cobre.

measuring ecosystem health.

Open questions to be addressed in the future include elucidating whether parental exposure to a given stressor could alter offspring tolerance to other substances, and evaluating potential interactions among multiple stressors that give rise to shifts in offspring trait values. On the other hand, the integration of vertical and horizontal routes for the transfer of pollutant effects among organisms may reveal new mechanisms behind community disruption and promote the generation of new ecological knowledge for the understanding of threatened ecosystems and hopefully for the development of more effective and enduring management actions.

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