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Gabrielle Ringot, Julien Gasparini, Marie Wagner, Marwan Cheikh Albassatneh, Adrien Frantz. More and smaller resting eggs along a gradient for pollution by metals: dispersal, dormancy and detoxification strategies in Daphnia?. Biological Journal of the Linnean Society, 2018, 124 (1), pp.11-20. 10.1093/biolinnean/bly026 . hal-01963571

HAL Id: hal-01963571
https://hal.science/hal-01963571

Submitted on 21 Dec 2018

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More and smaller resting eggs along a gradient for pollution by metals: dispersal, dormancy and detoxification strategies in Daphnia?

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Received 16 February 2018; accepted for publication 16 February 2018

Trace metals are bioavailable, persistent and potentially harmful chemicals commonly found in cities. In metal-polluted habitats, natural populations may evolve adaptations and may be composed of individuals exhibiting detoxification mechanisms, in particular through melanization, dispersal or dormancy abilities. Interestingly, Daphnia cyclically produce chitinous melanized envelopes called 'ephippia' encasing the resting eggs, which allow dispersal in space and in time (dormancy). Moreover, the success of dispersal decreases with increasing ephippial size. We hypothesized that populations living in polluted ponds produce more, darker and smaller ephippia than populations from unpolluted ponds. We sampled 51 ponds distributed in the Paris region and investigated the link between concentrations of seven trace metals and the presence of Daphnia and ephippia, and the size and pigmentation of ephippia. First, the presence of Daphnia was not linked to local metal concentrations, which ranged gradually from low to high values. Second, the probability of the presence of ephippia in sediments increased with metal concentrations, suggesting a selective advantage of Daphnia in producing dormancy stages in polluted habitats. Third, although ephippial pigmentation was not linked to metal concentrations, ephippial size decreased with increasing metal concentrations, suggesting a selection for increased dispersal in polluted habitats. Overall, our results show that anthropogenic pollution may have important microevolutionary consequences in urban populations, which are generally overlooked.

Keywords: ephippia – melanin – microevolution – size – trace metals.

Introduction

Urban areas are environments that concentrate human activities and associated forms of pollution, such as trace metals (Azimi et al., 2003). These chemical pollutants are bioavailable, bioaccumulative and persistent and have various and often toxic effects on reproductive output and survival (Møller, Forbes & Depledge, 1996; Žaltauskaitė & Sodienė, 2014; Rogalski, 2015). It is thus likely that they will affect the functioning and eco-evolutionary dynamics of ecosystems and that they may exert selective pressures leading to adaptations by organisms living in polluted environments (Klerks & Weis, 1987; Medina, Correa & Barata, 2007; Chatelain, Gasparini & Frantz, 2016).

Among potential adaptive phenotypic traits, melanin pigmentation may present an advantage owing to its detoxification properties. The chelation of metal ions by the several negative charges of melanin (McGraw, 2003) may promote organism detoxification by sequestering trace metals in melanized inert parts. Accordingly, a positive link between melanin pigmentation and trace metal pollution has been documented in several taxa (Loumbourdis & Vogiatzis, 2002; Fenoglio et al., 2005; Marques, Gonçalves & Pereira, 2008; Chatelain et al., 2014, 2016; Goiran, Bustamante & Shine, 2017). Therefore, more melanized individuals would have greater fitness than paler ones in the presence of toxic trace metals. Accordingly, previous studies in feral pigeons have shown that darker pigeons sequester more metals in their feathers and have higher juvenile survival when exposed to metals than less melanic ones (Chatelain et al., 2014, 2016). Thus, darker phenotypes may be more prevalent in habitats polluted by trace metals.

Adaptations to metal pollution may also include the ability to invest in the production of dispersal forms and/or dormancy stages. Dispersal is known to be selected when environmental variability in habitat quality is mainly spatial (Wiener &

Tuljapurkar, 1994; Bowler & Benton, 2005) and would make it possible to leave polluted habitats in favour of less polluted ones. Dormancy is favoured when temporal variability in habitat quality prevails (Hairston & Cáceres, 1996) and would make it possible to avoid pronounced pollution events until the return of optimal living conditions. Although the precise relative investment in dispersal or dormancy would thus depend on the spatial vs. temporal variability in pollution, the general feature of spatio-temporal heterogeneity of environmental pollution may therefore favour high dispersal and/or dormancy in polluted habitats.

In this study, we investigated whether metal concentrations were positively associated with the following: (1) the presence of dispersing and dormant stages; and (2) the intensity of melanin-based coloration of these stages in Daphnia. During its life cycle, this crustacean switches from parthenogenesis (clonal female reproduction) to sexual reproduction involving sexual females and males and resulting in up to two eggs. These eggs are protected by a chitinous envelope called the 'ephippium', which exhibits several interesting characteristics, such as variation in dispersal propensity, dormancy ability and melanin-based coloration. Indeed, ephippia can be dispersed by wind and by animals ('dispersal in space'; Maguire, 1963; Cáceres & Soluk, 2002; van de Meutter, Stoks & de Meester, 2008), and dispersal propensity decreases with ephippium size and buoyancy (van de Meutter et al., 2008). They can also remain dormant for several years or even decades ('dispersal in time'; Hairston et al., 1995; Cáceres, 1997, 1998). Finally, the ephippium is the only melanized stage in Daphnia (except in alpine and arctic populations, where active individuals are also melanic; Hebert & Emery, 1990), and this pigmentation is variable and heritable (Gerrish & Cáceres, 2003). Altogether, this set of traits offers a unique opportunity to test our hypotheses.

To this end, we sampled 51 ponds in the Paris region, measured the concentrations of seven trace metals (arsenic, cadmium, chromium, copper, nickel, lead and zinc) and correlated them with the presence of both Daphnia individuals and ephippia, and with the size and melanin pigmentation of the ephippia. According to the 'dispersal in space' and 'dispersal in time' hypotheses (Pietrzak & Ślusarczyk, 2006) and to the melanin 'detoxification' hypothesis (Chatelain et al., 2014, 2016), we predicted a higher production of ephippia and smaller, more melanized ephippia in more heavily polluted ponds.

Material & Methods

Sampling design

Fifty-one non-interconnected freshwater ponds and lakes were sampled on one occasion each in the Île- de-France (Paris region) between November 2015 and April 2016. Natural and artificial ponds were randomly chosen, according to their position and distance from the centre of Paris (Point Zéro des routes de France, 48° 51′ 12,24845′′ N, 2° 20′ 55,62563′′ E), to the elevation and to their depth and volume (calculated as half of an ellipsoid). Although we do not have information on either zooplankton assemblages or sedimentation rates, the high number of ponds (N = 51) sampled ensures that these factors are randomized and not confounded with the factors of interest in our study, making their influence on the variables very unlikely. For each pond, the presence of Daphnia individuals was recorded by vertically passing a net with a mesh size of 570 µm in the deepest part of the pond up to 50 cm. Regardless of the presence of Daphnia individuals in the water and at the same pond locations, we collected the top 3 cm of sediment in a circular area of 12 cm in diameter from each pond, representing ~3 years of egg accumulation in sediments (Hairston et al., 1995; Francis, 1997). To retrieve ephippia from the

sediments, we used the sugar centrifugal flotation method (Onbé, 1978; Cromar & Williams, 1991), which allows > 90% of the sampled eggs to be retrieved with one treatment (Onbé, 1978) without affecting egg via- bility (Onbé, 1978; Cromar & Williams, 1991), making it useful for future studies on these eggs.

Trace metal measurements

A sample of water was taken from each pond and filtered (0.45 µm mesh) in order to measure concentrations in seven trace metals [arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn)] considered to be primary freshwater micropollutants in the Île-de-France region (Nriagu & Pacyna, 1988; DRIEE, 2013). These metals originate both from industrial and urban direct effluent discharges and from total (wet and dry) atmospheric deposition (Nriagu & Pacyna, 1988; Azimi et al., 2003, 2005; DRIEE, 2013). This was a first-time sampling; therefore, we do not have historical data on metal pollution in the ponds, but we sampled ponds at increasing distance from the centre of Paris in order to obtain a pollution gradient that represents the variability of the Île-de-France region. We assumed that, despite their temporal variation, concentrations in water reflect recent exposure, including the period of release of ephippia. Concentrations were determined by mass spectrometry (ICP-MS Agilent 7500 cx) as described by Chatelain et al., 2016. All materials were cleaned and sanitized in 10% nitric acid baths to avoid trace metal contamination.

Characteristics of ephippia

For each ephippium, the area and the intensity score of melanin pigmentation (grey level of the ephippial surface) were measured via photographic analysis with ImageJ software (Gerrish & Cáceres, 2003). Photographs were taken with a

motorized microscope (SteREO Discovery.V12 Zeiss). Each ephippium was assigned to one of two Daphnia species groups, according to its shape and size characteristics: the *Daphnia galeata* and *Daphnia pulex* group (larger ephippia and 'narrowing sharply at posteroventral side') or the *Daphnia ambigua* and *Daphnia parvula* group (smaller ephippia and 'more or less symmetrical'; Vandekerkhove et al., 2004).

Statistical analyses

We investigated associations between pond characteristics (metal concentrations, longitude, latitude, elevation, distance from the centre of Paris, and pond volume) with a Pearson correlation matrix. Given that the concentrations of several metals appeared to be highly correlated, we performed all further statistics for each metal separately in order to avoid collinearity issues. We first used generalized linear models for binomial data to investigate the effects of metal concentration presence of Daphnia individuals and ephippia. Second, we used linear mixed models to test the effect of the metal concentration on ephippial size and melanin coloration, adding groups of species as a fixed effect and pond as a random effect to take pseudo-replication issues into account. For all analyses, metal concentration, ephippial size and melanin coloration were log10-transformed. Statistical analyses were performed with R version 3.3.1. We did not correct P-values for multiple testing, as suggested by García (2004), Moran (2003) and Nakagawa (2004).

Results

Environmental variables linked to metal concentrations

There was an important variability in concentrations of trace metals among ponds (Table 1). Concentrations of these seven trace metals in the ponds were

positively and significantly correlated (all $P \leq 0.03$, $0.31 \leq \rho \leq 0.76$), except between Zn and As ($P = 0.17$; Table 2). We found interesting correlations between environmental variables and metal concentrations. First, concentrations of Cr, Ni, Cu, Zn and Cd in water were lower when pond volume was high (Table 2). Second, concentrations of Cd, Cr and Ni were higher at higher elevation (Table 2).

Probability of the presence of Daphnia and ephippia

The probability of the presence of ephippia in pond sediments increased significantly with the concentration of Pb (logistic regression (LR) $\chi21 = 7.55$, d.f. = 1, $P = 0.006$; Fig. 1A), Cd (LR $\chi21 = 6.59$, d.f. = 1, $P = 0.01$; Fig. 1B) and Ni (LR $\chi21 = 4.50$, d.f. = 1, $P = 0.03$; Fig. 1C) but was not related to the concentrations of other metals (all $P > 0.32$). There appeared to be threshold concentrations in Ni, Cd and Pb (Ni, 3.61 µg/L; Cd, 0.056 µg/L; Pb, 1.45 µg/L) above which ephippia were systematically found in the ponds. The presence of Daphnia individuals was not dependent on trace metal concentrations (all $P > 0.29$). Pond size characteristics were not linked to the presence of ephippia and Daphnia individuals in ponds (all $P > 0.11$).

Ephippial characteristics

We did not find any statistical link between ephippial pigmentation and any of the metal concentrations (all $P > 0.07$). However, as the species group effect was significant for all metals (all $P \leq 0.0008$) we analysed ephippial size separately for each group species. In the *D. galeata* and *D. pulex* group, ephippia size decreased significantly with increasing concentrations of Pb ($\chi21 = 12.35$, d.f. = 1, $P = 0.0004$), Zn ($\chi21 = 5.96$, d.f. = 1, $P=0.015$),Cu($\chi21 =5.63$,d.f.=1,$P=0.018$)andCr ($\chi21 = 4.32$, d.f. = 1, $P = 0.038$; Fig. 2B–E), and such a tendency was observed for As ($\chi21 =$

3.28, d.f. = 1, P = 0.070) and Cd (χ21 = 2.83, d.f. = 1, P = 0.093; Fig. 2A, F). In the *D. ambigua* and *D. parvula* species group, a significant decrease in ephippial size with increasing concentrations of As (χ21 = 5.44, d.f. = 1, P = 0.019) and Pb (χ21 = 5.65, d.f. = 1, P = 0.017) was observed (Fig. 2A, B). Pond size characteristics were not linked to the size and pigmentation of ephippia (all P > 0.14).

Discussion

First, we found that concentrations of metals in ponds were variable at a local scale (Parisian region; Table 1, Fig. 3) and were positively correlated with one another (Table 2), as previously observed in various other environmental compartments [e.g. urban atmospheric deposition (Azimi et al., 2005), street dust (Charlesworth et al., 2003; Yongming et al., 2006)], suggesting common sources of these metals. Furthermore, these concentrations depended on the geographical and abiotic environmental factors measured (i.e. longitude, latitude, elevation, distance from the centre of Paris, and pond volume). This high heterogeneity in environmental pollution is likely to impact on populations and ecosystems.

In this context, we investigated whether the presence of both Daphnia individuals and ephippia was related to the levels of metallic pollution in ponds. Daphnia individuals were present all along the pollution gradient, even at the highest measured concentrations of metals (higher than the no observed effect concentration; Table 1). This is surprising given that Daphnia are considered to be highly sensitive to pollutants and are used as bioindicators (Tomasiks & Warren, 1996). This result suggests that Daphnia might have evolved the ability to survive sustainably in elevated concentrations (higher than the no observed effect concentration; Table 1) of metallic pollution; accordingly, cases of natural Daphnia populations adapted to elevated concentrations of metals have been documented

previously (Barata et al., 2002; Lopes, Baird & Ribeiro, 2006; Coors et al., 2009; Agra, Soares & Barata, 2011). Moreover, experimental evolution approaches have shown that Daphnia are able to adapt rapidly to elevated concentrations of metals (LeBlanc, 1982; Bodar et al., 1990; Hochmuth et al., 2015; Haap, Schwarz & Köhler, 2016). For instance, adaptation could involve the production of metallothionein, which binds metal ions and thus detoxifies the organism (Klaassen, Liu & Choudhuri, 1999; Haap et al., 2016). However, the precise phenotypic adaptations to elevated concentrations of trace metals remain unknown, and future studies should focus on their identification.

According to our predictions, we expected Daphnia to invest more in the production of diapausing ('dispersal in time' hypothesis) and dispersing stages, i.e. smaller ephippia ('dispersal in space' hypothesis), and in more melanized ephippia ('detoxification' hypothesis).

In agreement with our first prediction, the presence of ephippia in sediments was positively linked with the concentrations of three of the seven metals measured (Pb, Ni and Cd; Fig. 1). This result could be explained by various causes. First, as we hypothesized, higher production of ephippia could itself constitute an adaptation to adverse environments. Ephippia represent a diapausing stage that make it potentially possible to escape unfavourable conditions, such as temporarily dry (Roulin et al., 2013) or polluted ponds, until the return of optimal life conditions ('dispersal in time' hypothesis) and to maximize fitness in local habitats where the mortality rate of Daphnia individuals is high. This advantage of producing a dormancy stage in harsh environments has been proposed to account for a higher production of resting frost-resistant eggs by aphids in regions where low winter temperatures do not allow adult survival (Rispe et al., 1998). Second, the higher production of ephippia may allow them to leave a polluted patch, because the ephippium is the only dispersal stage in

Daphnia (‘dispersal in space’ hypothesis; Wiener & Tuljapurkar, 1994; Bowler & Benton, 2005). Alternatively, the higher production of ephippia in metal-polluted habitats could be adaptive, because the ability of ephippia to accumulate some metals (Wyn et al., 2007) could participate in pollutant sequestration out of the living tissues. However, our study is correlative, and we cannot exclude confounding effects in this positive association between ephippia and metal concentrations. Indeed, smaller ponds are more polluted (Table 2) but are also more temporal ponds that favour ephippial production (Roulin et al., 2013). Moreover, other non-tested factors are known to favour and/or initiate sexual reproduction in Daphnia, including exposure to solvents or endocrine disruptive compounds, pond temporality, parasite infection or predation (Hairston & Olds, 1984; Ślusarczyk, 1999; Zhang & Baer, 2000; Haeba et al., 2008; Roth et al., 2008; Baer, McCoole & Overturf, 2009; Roulin et al., 2013). Additionally, more ephippia could be found in more polluted habitats owing to the metal toxicity that would lower the hatching rate (Rogalski, 2015). Experimental approaches involving manipulation of metal concentrations are now called for.

Contrary to our second prediction, there was no relationship between the melanin coloration of ephippia and the concentrations of metals. However, we found that ephippia were smaller in more polluted ponds. This relationship could simply reflect the negative direct ecotoxicological effects of metals on the body length of adult Daphnia (van Leeuwen, Luttmer & Griffioen, 1985; Münzinger, 1990; Ward & Robinson, 2005; Agra et al., 2011) or an indirect ecotoxicological effect (e.g. through the food chain; Yan & Pan, 2002; Prosnier, Loreau & Hulot, 2015) and, subsequently, on the size of the ephippia they produce. More interestingly, as smaller ephippia have better success in dispersal (van de Meutter et al., 2008), the production of smaller ephippia could be selected in polluted habitats because it allows greater dispersal success. The differences we observed between metals in their relationships with life-

history traits of Daphnia might suggest that not all trace metals are in a toxic range of concentrations in these localities.

Altogether, our correlative work suggests that urban metal pollution may constitute an important environmental factor influencing natural populations, with regard to both ecotoxicological and microevolutionary aspects that need to be confirmed and investigated further through experimental approaches.

Acknowledgements

We thank the anonymous reviewers for their constructive comments on the manuscript. The metal measures of this study were performed with the assistance of Florence Le Cornec and Irina Djouraev on the platform Alysés at the Center IRD France-Nord (Bondy). We are grateful to the 'Mairie de Paris' for permission to take samples from ponds in Paris. We deeply thank John Holstead (senior lecturer, Lycée Fénelon, Paris) for language corrections.

Table 1. Trace metal concentrations in the sampled ponds of Ile-de-France, No-Observed Effect Concentrations (NOEC) and references.

Element	Range	Mean (µg/L)	SE[a]	NOEC[b] (µg/L)	References
Arsenic (As)	0.109 - 9.466	1.612	0.248	260	Biesinger and Christensen, 1972
Cadmium (Cd)	0.005 - 0.124	0.027	0.004	0.6	Kühn et al., 1989
Chromium (Cr)	0.063 - 3.095	0.623	0.099	0.00006	van Leeuwen et al., 1987
Copper (Cu)	0.041 - 56.560	4.136	1.182	11	Biesinger and Christensen, 1972
Nickel (Ni)	0.214 - 10.450	2.496	0.271	13	Kszos et al., 1992
Lead (Pb)	0.053 - 7.904	1.183	0.269	15	Biesinger and Christensen, 1972
Zinc (Zn)	0.658- 147.360	25.050	4.780	74	Biesinger et al., 1986

[a] SE indicates the standard error

[b] NOEC (21 days) determined on *Daphnia magna*

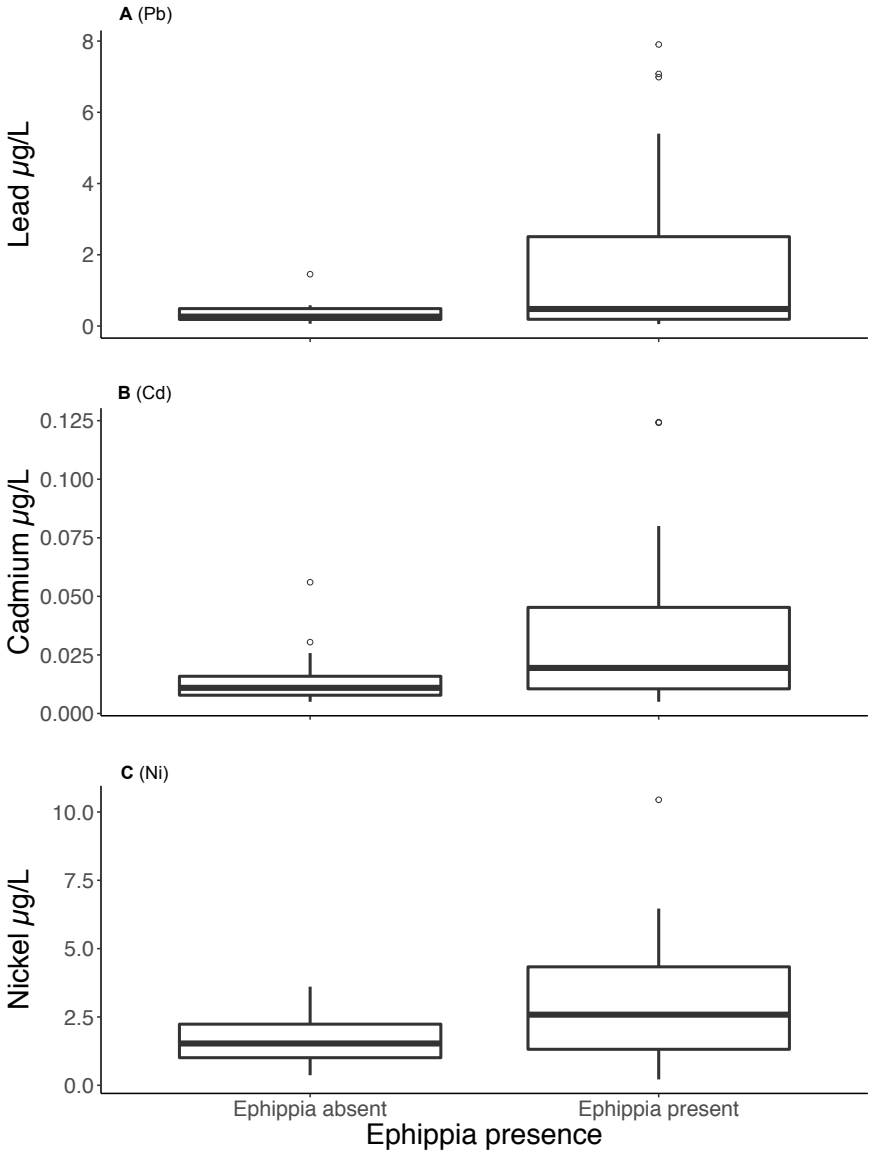


Fig. 1 Concentrations of metals in water ponds (µg/L) for (A) Lead (Pb), (B) Cadmium (Cd) and (C) Nickel (Ni) and ephippia presence.

Although relationships between ephippia presence probability and concentration of metals were tested using GLM with binary distribution, we present boxplots of metals concentrations among ponds according to ephippia presence/absence for better illustration purposes. Central bars represent median, boxes represent interquartile range, dots are outliers (> 1.5 × interquartile range).

Table 2. Pearson correlation matrix for environmental factors and trace metals concentrations of sampled ponds of Ile-de-France.

	Zn	Cd	Cr	Ni	Cu	As	Pb	Alt	Lat	Long	Dist	Vol
Zn		< 0.001	0.001	0.017	< 0.001	0.168	< 0.001	0.261	0.442	0.969	0.797	0.013
Cd	**0.68*** **		< 0.001	0.001	< 0.001	0.007	< 0.001	0.041	0.087	0.509	0.253	0.006
Cr	**0.45** **	**0.58*** **		< 0.001	< 0.001	0.018	< 0.001	0.001	0.144	0.774	0.084	0.016
Ni	**0.33***	**0.47** **	**0.56*** **		0.013	< 0.001	0.002	0.046	0.038	0.130	0.006	0.020
Cu	**0.71*** **	**0.65*** **	**0.48*** **	**0.35***		0.027	< 0.001	0.774	0.989	0.164	0.129	0.013
As	0.20	**0.37** **	**0.33***	**0.57*** **	**0.31***		< 0.001	0.255	0.746	0.225	0.534	0.222
Pb	**0.57*** **	**0.76*** **	**0.66*** **	**0.43** **	**0.59*** **	**0.52*** **		0.009	0.050	0.388	0.337	0.052
Alt	0.16	**0.29***	**0.43** **	**0.28***	0.04	0.16	**0.36** **		0.123	0.703	< 0.001	0.008
Lat	-0.11	-0.24	-0.21	**-0.29***	0.00	0.05	**-0.28***	-0.22		0.263	0.046	0.687
Long	0.01	-0.09	-0.04	-0.21	-0.20	-0.17	-0.12	0.05	0.16		0.336	0.777
Dist	-0.04	0.16	0.24	**0.38** **	-0.22	0.09	0.14	**0.68*** **	**-0.28***	0.14		0.804
Vol	**-0.35***	**-0.38** **	**-0.34***	**-0.32***	**-0.34***	-0.17	-0.27	**-0.37** **	0.06	-0.04	-0.04	

Given are correlation coefficients rho (below diagonal) and associated P-values (above diagonal). Alt: altitude, Lat: latitude, Long: longitude, Dist: distance from Paris, Vol: volume.

Significant correlations are in bold. * P<0.05; ** P<0.01; *** P<0.001

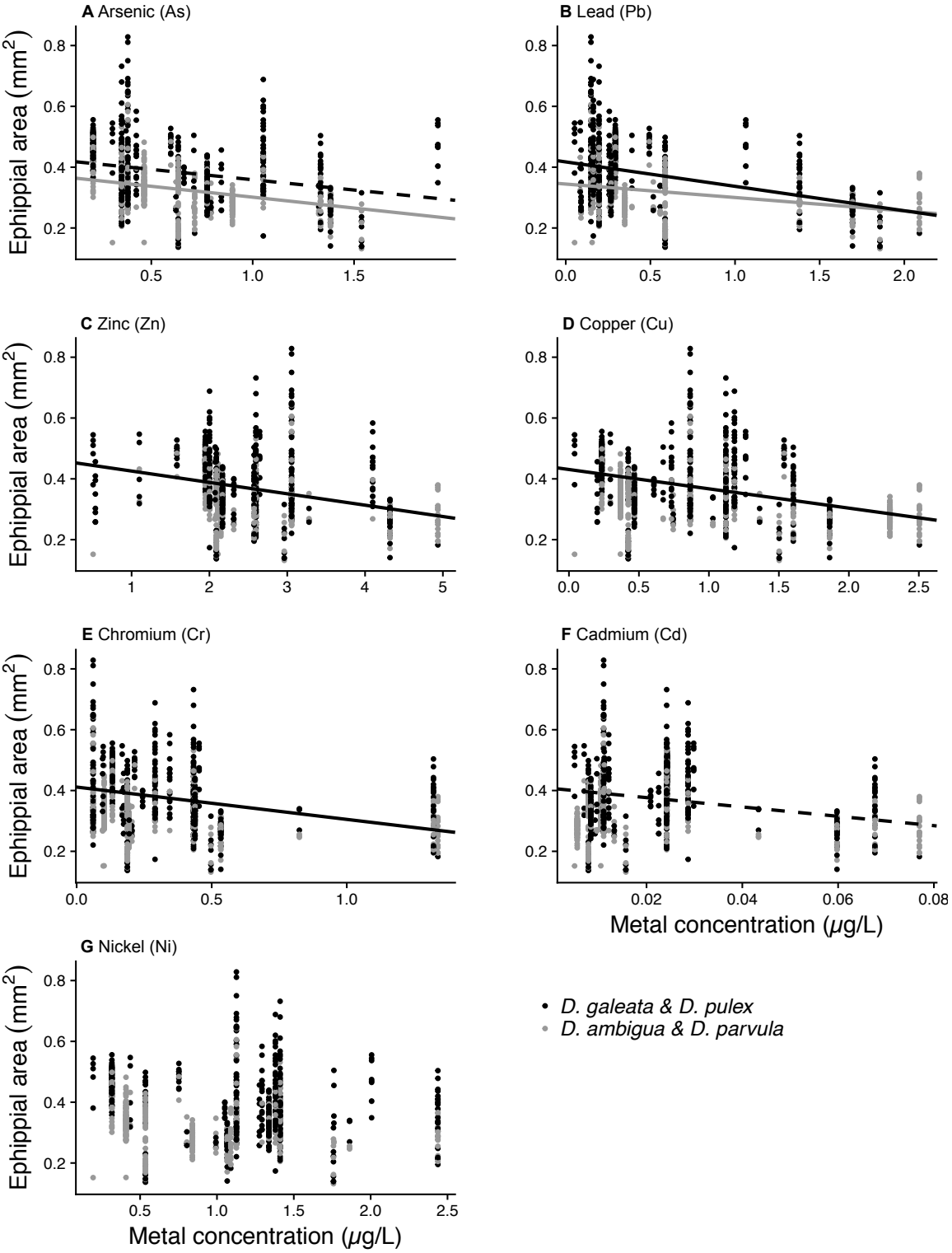


Fig. 2 Ephippial area (mm²) according to metals concentrations (µg/L) in water ponds.

Continuous lines show significant relationships; dashed lines show trend. Data for *D. ambigua & D. parvula* species group are in grey; data for *D. galeata & D. pulex* species group are in black.

Note that axes scales are log-transformed and that X axis has different scales for each metal.

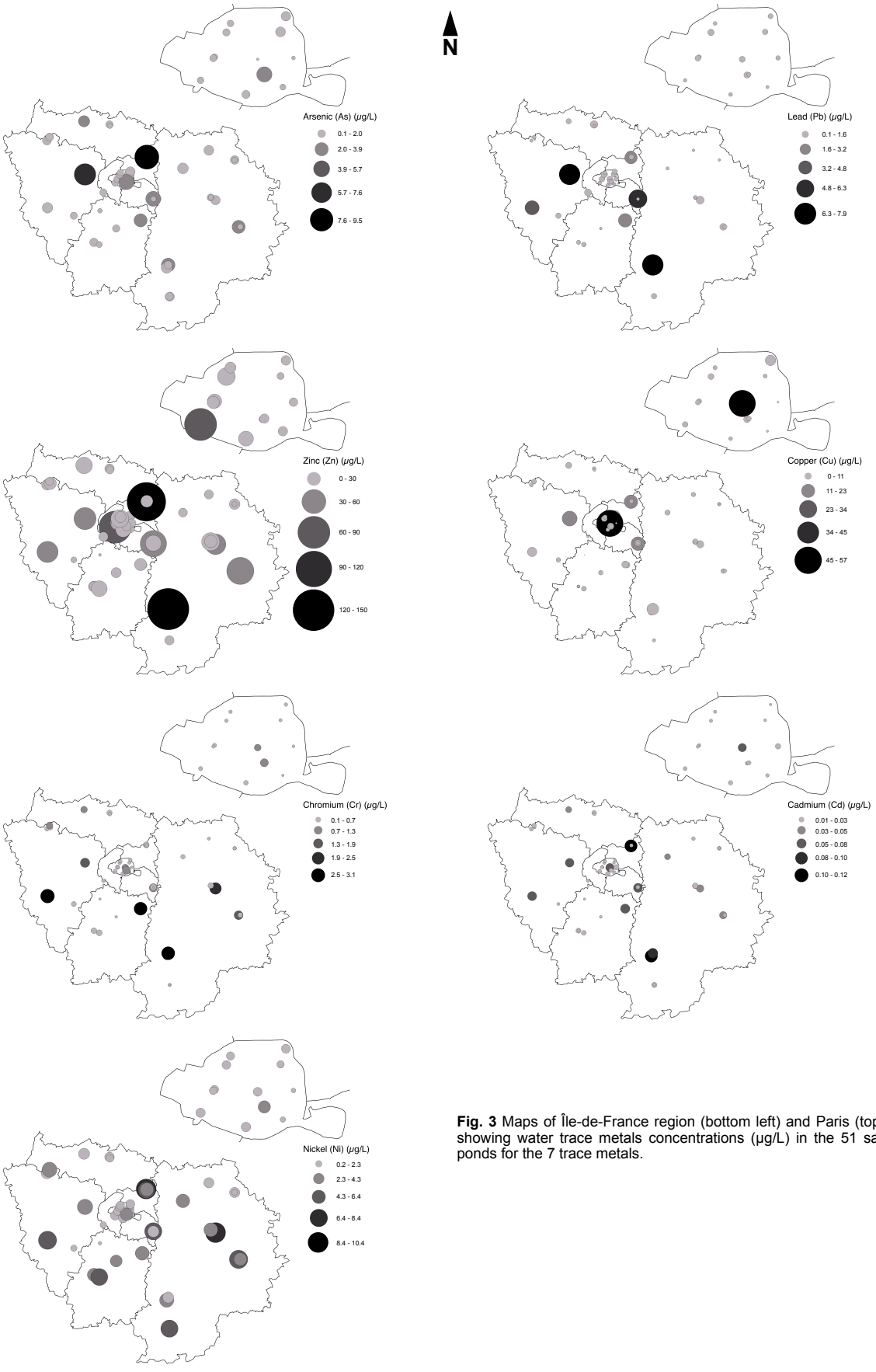


Fig. 3 Maps of Île-de-France region (bottom left) and Paris (top right) showing water trace metals concentrations (µg/L) in the 51 sampled ponds for the 7 trace metals.

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