In situ production of empty ephippia and resting eggs by an obligate parthenogenetic *Daphnia* population

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We directly analyzed the *in situ* production of ephippia and ephippial eggs by an obligate parthenogen *Daphnia pulicaria* population in a high-mountain lake by using sediment traps during the ice-free period. We recorded the number of eggs per ephippium collected in the traps and studied the zooplankton and phytoplankton communities as well as the nutrient conditions in the lake. Ephippia production was negatively correlated with the light phase of the photoperiod. Numerous empty ephippia were produced, and their number was negatively correlated with the epibiont burden of the chlorophyte *Korshikoviella gracilipes* on *Daphnia*. The number of healthy eggs per ephippium in the sediment traps was related to the edible phytoplankton biomass and/or epibiont burden. The proportion of empty ephippia was negatively related to the epibiont burden. These results suggest that the onset and rate of ephippia production is related to the photoperiod, and that the production of ephippial eggs per ephippium increases with greater food availability (phytoplankton biomass and/or epibiont burden). This is the first published evidence that food availability may favour resting egg reproduction under photoperiod conditions that trigger ephippia production.

KEYWORDS: *Daphnia pulicaria*; dormancy; ephippia; ephippial eggs; epibionts

INTRODUCTION

Many zooplankton taxa produce dormant propagules (diapausing eggs) in order to resist adverse conditions. When conditions in the water column are suitable, the hatching of these propagules allows diapausing organisms to resume normal development. Most studies on the induction of diapause in zooplankton have used *Daphnia*, a cladoceran that produces a diapausing egg pouch (ephippium) containing diapausing eggs. The genus *Daphnia* consists of cyclical parthenogens with sexual reproduction and obligate parthenogens with no sexual reproduction (Hebert *et al.*, 1988; Innes *et al.*, 2000). Sexuals bear higher immediate evolutionary costs in comparison with asexuals, because they need to produce both males and females and therefore pass only half of their genes to their offspring. However, although male production carries an energetic cost for sexuals, this can be compensated for by increased fecundity and/or survivorship with respect to obligate parthenogens (Innes *et al.*, 2000; Lehto and Haag, 2010; Lehtonen *et al.*, 2012), and sexual clones may be even better competitors than asexual ones (Wolinska and Lively, 2008). Hence, it is not clear that obligate parthenogenetic *Daphnia* has a major reproductive advantage over sexual *Daphnia*.

Obligate parthenogenesis is thought to be advantageous in arctic and alpine environments because of the short time available for reproduction in these habitats. In this situation, females hatching from dormant eggs can readily invest their resources in the production of dormant propagules rather than spending time on parthenogenesis to produce males that would then mate with females and generate resting eggs by meiosis (Dufresne *et al.*, 2011). The prevalence of asexuals at high latitudes and altitudes and in extreme environments, which has been acknowledged ever since the development of geographical parthenogenesis (Dufresne *et al.*, 2011), indicates their favourable performance in these marginal habitats (Ślusarczyk, 2009). Nevertheless, little is known about the reproduction mode of *Daphnia* at high altitudes (Dufresne *et al.*, 2011).

Zooplankton species need a stimulus to trigger the production of resting eggs, usually before the deterioration of environmental conditions, which can be signalled by photoperiod, population density, predator density, salinity or food, among other cues (Brendonck and DeMeester, 2003; Altermatt and Ebert, 2008). Photoperiod is considered the most important cue for seasonal diapause onset in crustacean zooplankton (Stross and Hill, 1968; Stross, 1987; Deng, 1996), but a major role has also been described for food quantity (Gyllström and Hansson, 2004). Thus, a low food concentration was found to favour dormancy initiation in temperate cladoceran

populations (Carvalho and Hughes, 1983; Ślusarczyk, 2001) and has been interpreted a poor environmental condition for their development. In contrast, Deng (Deng, 1996) found sexual reproduction to be more intense at high versus low food levels in *Daphnia pulicaria*, a cyclic parthenogenetic species. Hence, the influence of food on ephippial reproduction has not yet been fully elucidated.

It has been reported that ephippia may be produced empty in sexual (Zaffagnini, 1987; Cáceres, 1998; Keller *et al.*, 2007; Vaníčková *et al.*, 2010) and obligate parthenogenetic (Conde-Porcuna *et al.*, 2011) *Daphnia* populations. The production of empty ephippia may be due to empty laying or resting egg abortion, and elevated ephippial egg abortion rates were recently observed in an obligate parthenogenetic *Daphnia* population (Conde-Porcuna *et al.*, 2011).

The factors that control the abortion of resting eggs and/or the production of empty ephippia have not yet been established. In their study of sexual *Daphnia* populations, Keller *et al.* (Keller *et al.*, 2007) found a larger proportion of empty ephippia (reduced sexual fitness) in hybrid genotypes than in the parental taxon of *Daphnia galeata*. Food quantity and/or quality have been related to subitaneous egg abortion (Brooks, 1946; Richman, 1958; Boersma and Vijverberg, 1995); therefore, food availability may also affect the abortion of ephippial eggs and consequently the number of healthy ephippial eggs per ephippium. Moreover, although there have been numerous studies on the growth rate and resource allocation in *Daphnia*, little is known about the influence of nutrients such as P and N on ephippia production (Smith *et al.*, 2009).

Relatively few studies have attempted to directly document zooplankton diapausing egg production using traps that integrate periodic data (De Stasio, 1989; Cáceres, 1998; Jarnagin *et al.*, 2004). Most researchers studied the relevance of diapause egg-carrying females in late-season plankton samples without using traps (Carvalho and Wolf, 1989; Cáceres, 1998; Gliwicz *et al.*, 2001); hence, their occasional sampling may be incomplete. For this reason, the reported number of ephippial females may not reflect the true production of resting eggs over the whole period, because these may be aborted and the females may be predated, among other factors. A more accurate description of ephippial and resting egg production can be expected with the use of sediment traps.

Studies of *in situ* ephippial reproduction rates are commonly based on sexual *Daphnia* and consider the intensity of ephippia production (Cáceres, 1998; Cáceres and Tessier, 2004; Altermatt and Ebert, 2008; 2010), with less attention being paid to the number of diapausing eggs produced per ephippium (Kerfoot *et al.*, 2004). However,

the ephippial reproductive rate of *Daphnia* is likely to be overestimated if based solely on ephippial counts, without taking ephippial egg abortion or empty ephippia production into account (Conde-Porcuna *et al.*, 2011). This overestimation would be especially marked for systems in which the long-term persistence of *Daphnia* depends on ephippial egg production and hatching (Barea-Arco *et al.*, 2001; Pérez-Martínez *et al.*, 2007, 2013). Further studies are required that not only measure *in situ* ephippia production but also quantify the number of diapausing eggs per ephippium, analyzing the potential influence of environmental factors. More data are also needed on *in situ* ephippial reproduction and the production of empty ephippia in obligate parthenogenetic *Daphnia* populations. There have been only a few studies on the influence of environmental conditions (i.e. pool volume, hydroperiod length and number of desiccation events) on *in situ* ephippia production (Altermatt and Ebert, 2008, 2010), and no data from sediment traps are available on the possible effects of food availability on *in situ* ephippia production.

In the present study, we analyzed the *in situ* production of *Daphnia* ephippia in Río Seco, a high mountain lake. This cladoceran population is an obligate parthenogenetic lineage of the pulex complex and was designated *Daphnia pulicaria* for having the typical morphological features of this species. There are few or no overwintering females in this lake (Pérez-Martínez *et al.*, 2013). *Daphnia pulicaria* and its epibiont algae *Korshikoviella gracilipes* are major constituents of the simple plankton community in Río Seco Lake, and the epibiont dispersal stage is known to be actively and intensively grazed by *Daphnia* (Barea-Arco *et al.*, 2001). Consequently, the epibiont burden on *Daphnia* may be related to the food availability for this cladoceran, and Barea-Arco *et al.* (Barea-Arco *et al.*, 2001) suggested a mutualistic relationship between these species. We analyzed the number of healthy diapausing eggs per ephippium and paid special attention to the possible influence of environmental constraints on the production of *Daphnia* diapausing eggs. Pérez-Martínez *et al.* (Pérez-Martínez *et al.*, 2007) suggested that fixed abiotic factors (temperature and photoperiod) were the main cues for switching to *Daphnia* diapause (end August-beginning September) in Río Seco Lake. A laboratory experiment on *Daphnia* from Río Seco Lake showed that, after the onset of ephippia production, they were produced by well-fed animals or by starving epibiont-infected animals but not by starving non-infected animals (Barea-Arco *et al.*, 2001). Based on these results, we hypothesized that, after the photoperiod-induced onset of ephippial reproduction, it may be influenced by other variables, such as food availability (algal density and/or epibiont infection level on *Daphnia*).

METHOD

Study area

Río Seco is a small (0.4 ha with northeast-southwest orientation), oligotrophic and shallow ($z_{max} = 2.90$ m) high-mountain lake (3020 m.a.s.l.) of glacial origin located in the Sierra Nevada mountains (southern Spain) above the tree-line on siliceous bedrock. The soils are very poorly developed and do not support agriculture or forestry use (Morales-Baquero *et al.*, 2006). This fish-free lake has a catchment area of 9.9 ha (Morales-Baquero *et al.*, 2006) and is ice and snow-covered during 6–8 months of the year.

Data have been published on the physico-chemical characteristics of the lake in different years (Barea-Arco *et al.*, 2001; Morales-Baquero *et al.*, 2006; Pérez-Martínez *et al.*, 2013). During the ice-free period, the Secchi disk visibility exceeds the water depth, the lake is not thermally stratified (see thermistor data in García-Jurado *et al.*, 2011), and the maximum temperature is 16–18° C. Dissolved organic carbon values range from 62.1 to 283.5 μmol L^{-1}, pH values from 6 to 7.4 and acid neutralizing capacity values from 0.05 to 0.20 meq L^{-1}. The lake has no clearly differentiated littoral zone, but its shoreline is dominated by bryophytes (*Drepanocladus fluitans*).

Data on the wind conditions were analyzed to examine the potential influence of sediment resuspension on the number of ephippia recorded in the traps (Kerfoot *et al.*, 2004; Kelderman *et al.*, 2012). The average wind speeds and directions during the study period were gathered from a meteorological station (Refugio Poqueira, Red Guadalfeo) belonging to the Andalusian Regional Government (Spain) and managed by the Fluvial Dynamics and Hydrology Research Group of the Universities of Cordoba and Granada (Spain). This station is near Río Seco Lake (ca. 3 km southeast) and located above the tree-line at 2510 m.a.s.l. on the same south face of Sierra Nevada as Río Seco Lake. It provides information every 5 min.

Rio Seco Lake contains chlorophyll *a* concentrations <2 μg L^{-1} and a low phytoplankton biomass (<200 μg C L^{-1}). The phytoplankton community is mainly composed of *Chromulina* spp., *Synechococcus nidulans*, *Dictyosphaerium chlorelloides* and zoospores of the epizoan chlorophyte *Korshikoviella gracilipes* (Barea-Arco *et al.*, 2001; Pérez-Martínez *et al.*, 2007). The green alga *Korshikoviella gracilipes* regularly attaches to crustacean zooplankton in this lake, especially to *Daphnia pulicaria* (Pérez-Martínez *et al.*, 2001).

Korshikoviella gracilipes has a complex life history, and its sexual life cycle comprises five different stages: zoospore, chlorangioid, ankyroid, adult and cyst (Barea-Arco *et al.*, 2001; Pérez-Martínez *et al.*, 2001). The zoospores (dispersal stage of the epibiont life cycle) are biflagellate cells

living as free-swimming planktonic organisms, attaching their apical end to substrate organisms encountered. This unicellular attached stage (chlorangioid) then develops into the ankyroid, a bicellular organism, by transverse division and cell elongation. Ankyroids reach the adult stage, the primary reproductive phase of the *K. gracilipes* life cycle, by elongation through transverse divisions. Each cell of the adult organism is able to produce zoospores by mitotic division.

The zooplankton community is dominated by the calanoid *Mixodiaptomus laciniatus* and the cladoceran *Daphnia pulicaria*, which together comprise more than 90% of the total zooplankton biomass. Little contribution to the zooplankton density and biomass is made by the other copepod or cladoceran species (*Diaptomus cyaneus, Acanthocyclops vernalis, Bosmina longirostris*) or by rotifer species (*Hexarthra bulgarica, Euchlanis dilatata, Trichocerca tenuior*) (Barea-Arco *et al.*, 2001). In Río Seco Lake, *D. pulicaria* generally reproduces by subitaneous eggs until mid-August, when there is a shift to ephippial egg production; however, there is usually little reproduction by subitaneous eggs in this lake (Pérez-Martínez *et al.*, 2007, Conde-Porcuna *et al.*, 2011).

Barea-Arco *et al.* (Barea-Arco *et al.*, 2001) observed that the chlorangioid stage of *K. gracilipes* was attached to all of the zooplankton species in Río Seco Lake, but mainly to *D. pulicaria*. The ankyroid and adult stages were found almost exclusively on the legs of *D. pulicaria*, indicating that *K. gracilipes* only completes its life cycle on this cladoceran species. The attached stages of the epibiont are limited to the crustacean inter-moult period.

Field samples

Zooplankton was collected over the ice-free period in 2005, dividing the lake into five areas of about the same size and collecting a sample at a random point within each area. A tube sampler (6.7 cm diameter) was used to collect the whole water column in an integrated sample. Zooplankton was gathered from 5 L of this homogenized volume of water from each sampling point, yielding a total of 25 L of water for zooplankton analysis on each sampling date. Zooplankton was filtered through a 40-μm mesh and immediately fixed with 70% ethanol following the method of Black and Dodson (Black and Dodson, 2003).

Phytoplankton (100 mL) was collected at the deepest point of the lake using the same methodology as for zooplankton and was immediately fixed with Lugol's solution. Water for chemical analysis was collected as an integrated sample taken from the deepest point of the lake, and its temperature was measured with a Waterproof PC300 meter.

Zooplankton and phytoplankton samples were allowed to settle in counting chambers before examination under a Nikon inverted microscope according to the technique of Utermöhl (Utermöhl, 1958). We measured the length of *Daphnia* individuals from the base of the tail spine to the top of the head (length of juveniles <1500 μm). We counted ephippia and resting eggs in 1626 individuals collected during the study period.

Algal samples were analyzed for species identification and cell density, measuring at least 30 cells of each phytoplankton species. Phytoplankton biovolume was estimated by using geometrical formulae (Hillebrand *et al.*, 1999) and was converted to carbon biomass following Menden-Deuer and Lessard (Menden-Deuer and Lessard, 2000).

The zooplankton was examined for *K. gracilipes* epibionts at each sampling point on each sampling day, focusing on *D. pulicaria* and examining a total of 1626 individuals. Epibiont prevalence, defined as the percentage of zooplankton individuals with epibionts (Willey *et al.*, 1990), was calculated separately for chlorangioids and for ankyroids/adults on each infected *Daphnia* individual. Scales were used to assess the epibiont burden, defined as the number of epibionts found on a substrate organism (Threlkeld *et al.*, 1993). A 6-point scale (0 = no presence of epibionts to 5 = extremely heavy infestation) was used for the chlorangioid burden (Barea-Arco *et al.*, 2001) and a different 6-point scale (0 = no cells, 1 = 1–50 cells, 2 = 50–100 cells, 3 = 100–200 cells, 4 = 200–500 cells, and 5 = >500 cells) for the ankyroid/adult burden. Hereafter, the terms "prevalence" and "burden" refer to chlorangioids and the terms "adult prevalence" and "adult burden" to ankyroids/adults.

Dissolved nutrient concentrations were analyzed after filtering water through Whatmann GF/C filters, determining soluble reactive phosphorus (SRP) according to Murphy and Riley (Murphy and Riley, 1962), analyzing total phosphorus (TP) after persulfate digestion and determining nitrate (NO_3), ammonium and total nitrogen (TN) according to APHA (APHA, 1992). Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate and ammonium (Weithoff *et al.*, 2001).

Sediment traps

Two twin sediment traps were placed at a random point within each of the five lake study areas from 21 July until the lake started to freeze on 3 November 2005 (11 samples from sediment traps were obtained during this period). Sediment traps, i.e. plastic cylinders of 31 cm height and 2.25 cm radius (de Vicente et al., 2008), were placed at 0.5 m from the bottom of the lake and sampled weekly. The tops of the traps were located at several depths between 44 and 105 cm. The trap content was

filtered through a 30 μm mesh and immediately preserved with 70% ethanol. *Daphnia* ephippia in the traps were counted in the laboratory. Each ephippium was then opened with a pair of dissecting needles, and the healthy eggs (i.e. those with no signs of degeneration) were then counted (Cáceres, 1998). The mean count for the two cylinders in each twin trap was considered in the analysis.

Statistical analysis

STATISTICA (Statsoft) and R 2.14.0 (R Foundation for Statistical Computing) programs were used for the statistical analysis. Normality was checked with the Kolmogorov−Smirnov test, and non-normal data were normalized by logarithmic transformation or by arcsine−square-root transformation (Sokal and Rohlf, 1995). A two-way ANOVA without replication was used to analyze temporal and spatial variations in the number of *Daphnia* ephippia and healthy ephippial eggs collected in the sediment traps. Partial correlation coefficients were used to explore the potential influence of environmental variables (mainly photoperiod, temperature, nutrient concentrations, edible algae biomass, epibiont prevalence and burden and *Daphnia* density) on the production of ephippia and ephippial eggs. Environmental variables were selected by a stepwise forward multiple regression procedure. The normality of residuals was checked by the Shapiro test, and outliers were examined by using the ± 2 sigma limits (Feinstein, 1996). Durbin−Watson analyses of temporal autocorrelation indicated non-critical autocorrelation.

Wind speeds and directions were also considered in related analyses to study whether wind resuspension may have affected the numbers of ephippia collected in the traps. The vector-averaged wind direction for each period, a circular histogram and circular−linear correlations were obtained by using the Oriana program (Kovach Computing Services). In order to accommodate the circular covariate wind direction, the sine and cosine of wind direction was included in the stepwise forward multiple regressions rather than the wind direction variable itself (Johnson and Wehrly, 1978; Mardia and Jupp, 2000; Jammalamadaka and Lund, 2006).

RESULTS

Field samples

The lake became totally ice-free at the beginning of May, after a thawing period of approximately 14 days (Fig. 1A). The zooplankton community was dominated by copepod nauplii at the beginning of the ice-free period, while adults of *D. pulicaria* were more important at the end of the growing season (Fig. 1A). This zooplankton density pattern has been observed in previous years (Fig. 3 of Barea-Arco *et al.*, 2001). A small number of empty *Daphnia* ephippia was observed in lake samples during June (0.03 ephippia L^{-1}). Temperature was lower than 18°C throughout the study period (Fig. 1A).

Edible phytoplankton biomass [all species with a longest geometric axis (GALD) <40 μm] was below 20 μg C L^{-1} throughout the ice-free period (Fig. 1B). Zoospores of *K. gracilipes* became the dominant taxon during autumn (Fig. 1B), as previously observed by Barea-Arco *et al.* (Barea-Arco *et al.*, 2001).

There was a significant correlation between daily *K. gracilipes* zoospore (log-transformed) and *D. pulicaria* densities ($r = 0.57$, $P = 0.03$, $n = 15$). Prevalence was also significantly correlated with burden ($r = 0.86$, $P < 0.01$ for chlorangioids; $r = 0.83$, $P < 0.01$ for adults). In the multiple regression analyses, the *D. pulicaria* chlorangioid burden and adult burden were negatively correlated with the concentration of TN (Fig. 2), but no significant relationships were observed between the epibiont burden and the other environmental conditions (photoperiod, temperature, TP, SRP, NO$_3$).

Additional forward regression analysis showed that the proportion of ephippial females in lake samples during the period in which sediment traps were in place was solely related to the photoperiod ($r = -0.91$, $P < 0.001$, $n = 11$), whereas neither temperature nor food availability, among other factors, were significant in the regression analysis.

Sediment traps

The largest number of ephippia with eggs in the sediment traps was observed during the autumn, just before the freezing of the lake, corresponding with the highest densities of ephippial females with at least one egg (Fig. 3). The presence of *Daphnia* ephippia with eggs in sediment traps was detected on 22 August, although empty ephippia started to be recorded in the traps 3 weeks earlier. The total annual production was close to 4000 healthy resting eggs m^{-2}, with a mean of less than one healthy resting egg per ephippium (Table I). More than 70% of released ephippia were empty.

During the period in which ephippial females were observed in the lake, no differences were found in the total number of eggs per ephippium between those obtained from the sediment traps and the lake samples (Table I; paired *t*-test, $t = 1.09$, $P = 0.34$, $n = 5$). Moreover, the proportion of empty ephippia detected in the ephippial females from the lake samples was lower
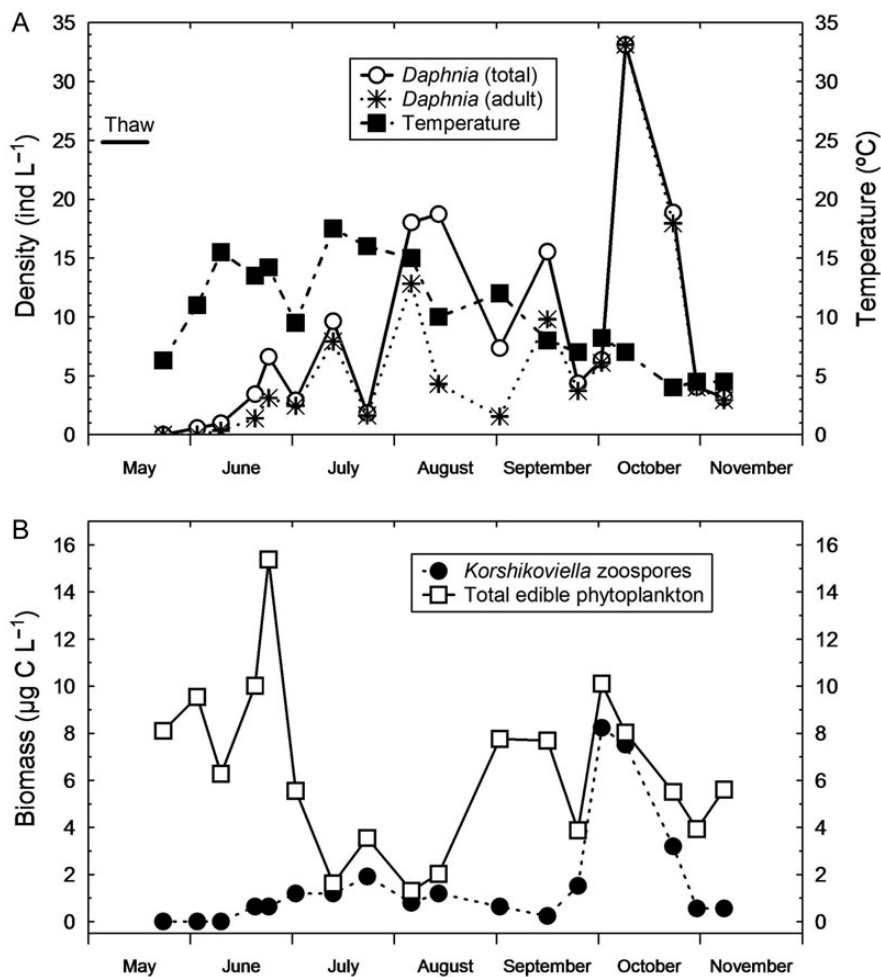
Fig. 1. (**A**) Abundance of *Daphnia pulicaria* during the ice-free period (daily mean of values from five samples of 5 L of water each). Temperature values are also given. (**B**) Biomass of edible phytoplankton and zoospores of *K. gracilipes* during the ice-free period.
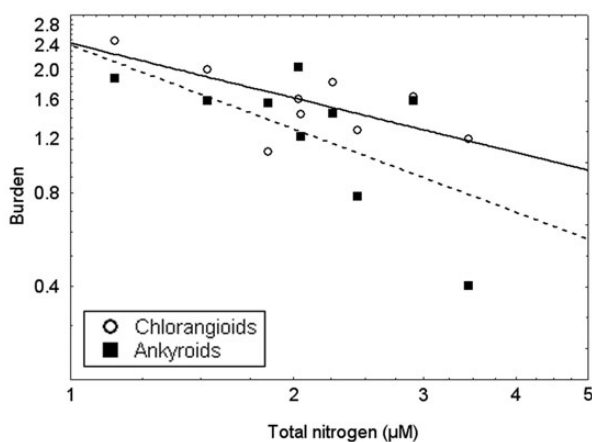
Fig. 2. Relationship of the chlorangioid (Pearson's $r = -0.77$, $P < 0.01$) and ankyroid burden (Pearson's $r = -0.69$, $P < 0.05$) with the concentration of TN.
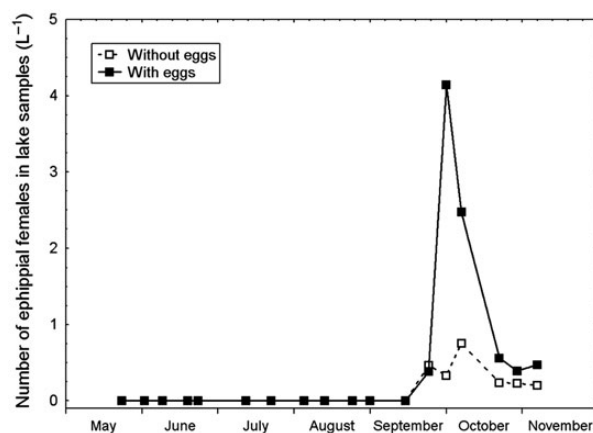
Fig. 3. Mean numbers of ephippial females in the lake during the study year.

Table I: Ephippia and resting egg production per year registered in the sediment traps and ephippial data from lake samples

	Production (per trap), mean ± SE	Production (m²), mean ± SE	Data from lake samples, mean ± SE
Total ephippia	12.1 ± 0.7	7610.1 ± 460.0	
Total ephippia (female^{-1})[a]			0.20 ± 0.03
Proportion of empty ephippia	0.731 ± 0.07	0.731 ± 0.07	–
Proportion of empty ephippia (in each sampling day)[a]	0.65 ± 0.07	–	0.30 ± 0.08
Total ephippial eggs	6.2 ± 1.6	3899.4 ± 997.4	–
Total ephippial eggs (female^{-1})[a]			0.19 ± 0.05
Total ephippial eggs per ephippium	0.54 ± 0.15	0.54 ± 0.15	–
Total ephippial eggs per ephippium (on each sampling day)[a]	0.73 ± 0.21	–	0.96 ± 0.12
Healthy ephippial eggs	5.8 ± 3.7	3647.8 ± 1050.5	–
Healthy ephippial eggs per ephippium	0.499 ± 1.7	0.499 ± 0.155	–

Data are referred to the period July to November except where indicated.
[a]Only data from September to November are included to compare traps and lake samples (no ephippial females were observed in lake samples before these dates).
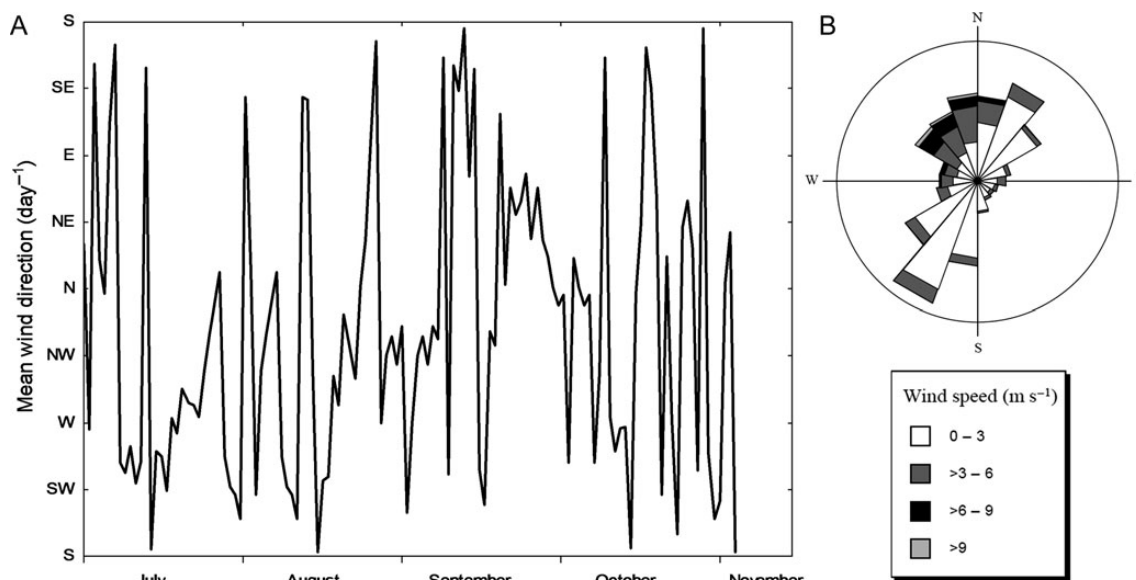

Fig. 4. (**A**) Mean wind direction over the sediment trap monitoring period (July–November) in Sierra Nevada (Spain). (**B**) Raw data on wind direction and speed throughout the study period (5′ intervals).

than the proportion found in the traps (Table I; paired *t*-test, $t = 2.96$, $P = 0.041$, $n = 5$).

The wind direction during the summer was predominantly from the northest or southwest (Fig. 4). Under storm conditions and at higher wind speeds, north and northwest directions were highly predominant, even exclusive. No relationship was found between wind direction patterns and the quantity of ephippia in the traps (Figs 4 and 5). Circular–linear correlation analysis showed no association between wind direction and the number of ephippia with eggs ($r = 0.42$, $P = 0.24$, $n = 11$) or without eggs ($r = 0.31$, $P = 0.47$, $n = 11$). Moreover, the average daily wind speed showed no relationship with the number of sedimented ephippia in the traps (with or without eggs) (Fig. 5) and was not significantly correlated with the number of ephippia ($r = -0.39$, $P = 0.23$, $n = 11$), or the proportion of empty ephippia ($r = 0.08$, $P = 0.83$, $N = 10$). Likewise, when the speeds of winds blowing along the main axis of the lake (northeast–southwest) were considered alone, they also showed no significant correlation with the number of ephippia ($r = -0.50$, $P = 0.11$, $n = 11$) or the proportion of empty ephippia ($r = 0.45$, $P = 0.19$, $n = 10$).

The number of ephippia and ephippial eggs found in the traps increased over time but no difference was observed among lake areas (Table II); the total number of
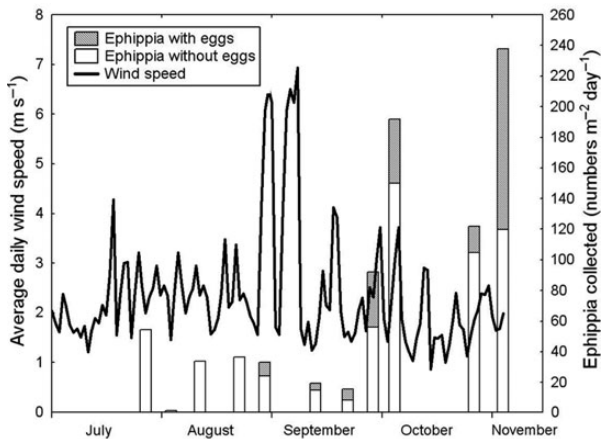
Fig. 5. Average daily wind speeds from July to November, and ephippia collected in the sediment traps.

Table II: Effects of the lake zone and time (days) on the ephippia and healthy eggs collected in the sediment traps (two-way ANOVA without replication)

	df	MS	F	P
Healthy ephippial eggs				
Lake zone	4	0.175	0.860	0.504
Time	5	0.945	4.635	0.005**
Error	20	0.204		
Total	29			
Number of ephippia				
Lake zone	4	0.343	0.538	0.709
Time	9	9.738	15.289	0.000***
Error	36	0.637		
Total	49			

$**P < 0.01$.
$***P < 0.001$.

ephippia was negatively related to photoperiod ($r = -0.77$, $P < 0.01$, $n = 11$), which was the only variable selected by the forward stepwise selection procedure, in which the temperature, nutrient availability, wind speed and direction, chlorangioid and ankyroid burden and *Daphnia* density were also considered.

Stepwise multiple regressions showed that the total number of ephippial eggs in sediment traps and the number of healthy resting eggs were both significantly related to the concentration of edible phytoplankton and to the photoperiod (Table III). Finally, the number of healthy resting eggs per ephippium and the proportion of empty ephippia were significantly related to the epibiont burden on *Daphnia* (Table III, Fig. 6). The proportion of empty ephippia was negatively correlated with photoperiod, but only the epibiont burden on *D. pulicaria* proved significant in the forward stepwise regression analysis (Fig. 6B), although the DIN:SRP ratio showed a

Table III: Multiple regression analyses of diapausing reproductive traits of Daphnia pulicaria *in 2005 (recorded in sediment traps) and the following independent variables: temperature, photoperiod, nutrient availability (TP, TN, SRP, DIN), edible phytoplankton biomass, chlorangioid and ankyroid burden, and density of* Daphnia

	Partial correlation coefficients		
	EE ($n = 9$)	HEE ($n = 9$)	HEE ratio ($n = 8$)[a]
Photoperiod	−0.86**	−0.79*	
Edible phytoplankton biomass (log)	0.90**	0.89**	
Burden (chlorangioids) (log)			0.94***
Adj. R^2	0.92***	0.89***	0.88***

Variables were selected by a stepwise forward procedure and the partial correlation coefficients are given (Sokal and Rohlf, 1995).
EE, total ephippial eggs (log + 1); HEE, healthy ephippial eggs (log + 1); HEE ratio, healthy ephippial eggs per ephippium (log + 1); Adj. R^2, adjusted coefficient of determination of the multiple regression model.
[a]One outlier was removed, but a similar result was obtained when it was included (see Fig. 6A).
$*P < 0.05$.
$**P < 0.01$.
$***P < 0.001$.

negative trend ($r_p = -0.77$, $P = 0.076$). Neither wind speed (total mean or northeast–southwest mean or maximum value) nor wind direction proved significant in this forward regression analysis.

DISCUSSION

This direct study of *in situ* ephippia and ephippial egg production by *Daphnia* contributes the first evidence of the possible influence of food conditions on the number of eggs per ephippium and the release of empty ephippia. In most previous studies on *in situ* ephippial production, the number of ephippia was used as an indicator of the reproductive rate (Cáceres, 1998; Cáceres and Tessier, 2004; Altermatt and Ebert, 2008, 2010). However, experimental findings of ephippial egg abortion in *D. pulicaria* from Río Seco Lake suggested that the ephippial reproduction has been overestimated, because the number of healthy eggs per ephippium can be much lower than the two eggs assumed in previous studies; therefore, most of the empty ephippia released by *Daphnia* may result from the abortion of resting eggs (Conde-Porcuna *et al.*, 2011).

According to our results, photoperiod may be the main factor influencing *in situ* ephippia production. In their study of Rio Seco Lake, Pérez-Martínez *et al.* (Pérez-Martínez *et al.*, 2007) suggested that fixed abiotic
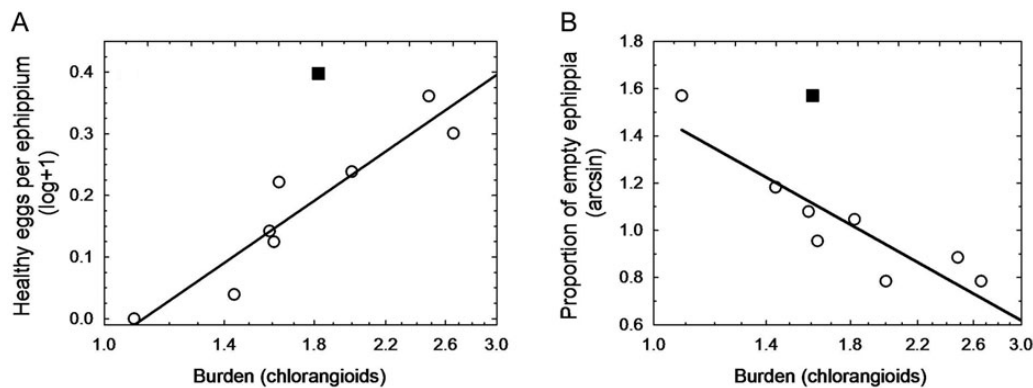
Fig. 6. (**A**) Relationship between the number of healthy eggs per ephippium recorded in sediment traps and the chlorangioid burden on *Daphnia*. See Table III for statistical data. (**B**) Relationship between the proportion of empty ephippia (arcsine–square-root transformation) and the chlorangioid burden ($r = -0.90**$ removing one outlier; $r = -0.79*$ including all data), which was the only significant variable selected by a forward stepwise procedure. ■: outliers. $*P < 0.05$, $**P < 0.01$.

factors (temperature and photoperiod) were the main cues for switching to diapause, but photoperiod appeared to be the sole relevant factor in the present study. Photoperiod has been described as the most important cue for diapause onset in crustacean zooplankton (Stross and Hill, 1968; Stross, 1987; Deng, 1996), although other environmental features can be involved, including water temperature, predation risk or density-dependent factors such as crowding and food availability (Stross and Hill, 1965; Hairston and Kearns, 1995; Slusarczyk, 1999; Yurista and O'Brien, 2001). There is no predation risk in Rio Seco Lake, and *Daphnia* density showed no relationship with ephippia onset in the present study, but food availability (epibiont burden and/or edible phytoplankton biomass) was related to ephippia reproduction.

The presence of empty ephippia is considered a common phenomenon in sexual reproduction in *Daphnia* (Keller *et al.*, 2007). The large proportion of empty ephippia in the field can be attributed to the failure of some ephippia-producing *Daphnia* females to mate before the next moulting, when unfertilized ephippia are shed (Wolinska and Lively, 2008). Although empty ephippia have also been found in obligate parthenogens (Wolinska and Lively, 2008; Conde-Porcuna *et al.*, 2011), the obligate parthenogenetic *Daphnia* has no mating constraints and are not expected to produce a high proportion of empty ephippia. However, in an experimental study, Conde-Porcuna *et al.* (Conde-Porcuna *et al.*, 2011) reported high abortion rates and/or low production of eggs per ephippium in an obligate pathenogenetic *Daphnia pulicaria*. The large number of ephippia without eggs may be attributable to resource limitation (Wolinska and Lively, 2008), because ephippia require a greater investment of energy in comparison with directly developed offspring. Lynch (Lynch, 1983) demonstrated that the production of an ephippium is energetically expensive (equivalent to the

expenditure for nine parthenogenetically produced eggs), and Gilbert (Gilbert, 2010) reported that rotifers need higher food concentrations to produce resting instead of amictic eggs.

Barea-Arco *et al.* (Barea-Arco *et al.*, 2001) observed ephippial production by *D. pulicaria* in Río Seco Lake when food was available but not under starvation conditions (no food or epibiont algae). After induction of ephippia production by environmental factors other than food quantity, a greater food supply may increase the number of ephippial eggs released, which is of special importance in oligotrophic lake ecosystems like Río Seco Lake, with short growth and reproduction periods and only intermittent winter populations. The experiment by Barea-Arco *et al.* (Barea-Arco *et al.*, 2001) also showed that unfed *Daphnia pulicaria* infected by *Korshikoviella* were able to survive and reproduce. Consequently, a high epibiont burden on *Daphnia* should indicate high food availability for this cladoceran, because the epibiont dispersal stage is actively and intensively grazed in Río Seco Lake (Pérez-Martínez *et al.*, 2001). *Daphnia* may feed on *K. gracilipes* zoospores produced by adult epibionts attached to the cladoceran legs as well as on zoospores detached from the body of *D. pulicaria*. *Korshikoviella gracilipes* can abandon the substrate organism (*Daphnia*) before, after or during moulting, although the number of zoospores released and/or detached from *Daphnia* is much higher just before moult, at up to 1800 zoospores min^{-1} (Pérez-Martínez *et al.*, 2001). Hence, there are pulses of high food concentration in Río Seco Lake that coincide with the *Daphnia* moulting period, which may favour an increase in the number of resting eggs per ephippium. Estimation of the food availability for *Daphnia* in this lake must consider not only the algae concentration but also the epibiont burden. Account should also be taken of the negative effect that epibiont algae may exert on the

proportion of released empty ephippia (Fig. 6). In this regard, the present findings indicate that a higher edible phytoplankton concentration and epibiont burden may enhance ephippial reproduction and may also increase the number of healthy ephippial eggs, given the larger number of released ephippial eggs (in absolute terms and per ephippium) at higher food concentration and/or greater epibiont burden (Table III). These results support the hypothesis that, after the onset of ephippia production, healthy ephippial eggs are produced by well-fed or epibiont-carrying animals.

In contrast to our findings, most studies of sexual *Daphnia* populations have reported a higher cladoceran ephippial production at lower food levels (Carvalho and Hughes, 1983; Kleiven *et al.*, 1992; Ślusarczyk, 2001). LaMontagne and McCauley (LaMontagne and McCauley, 2001), in another sexual *Daphnia* population, showed that a reduction in food levels produces a qualitative change in the reproductive strategy (to sexual reproduction) of matured offspring but not of adults. Alekseev and Lampert (Alekseev and Lampert, 2001), in an obligate parthenogenetic *D. pulicaria* strain, found ephippial production at low food concentrations but none at high food concentrations, regardless of day-length conditions.

Regardless of whether the ephippia are produced by sexual or asexual reproduction, low food concentrations or a reduction in food levels appear to trigger their production. However, if their production is induced by other signals (e.g. photoperiod), a high food concentration may then increase the number of ephippial eggs, as indicated by the present results, ensuring an adequate number of eggs for recruitment and lake colonization in the following year. In a study of a cyclically parthenogenetic *D. pulicaria* population, a higher number of ephippia per female was observed with greater food availability once sexual reproduction had been triggered by the critical photoperiod (Deng, 1996), consistent with our findings in an obligate parthenogenetic population.

We also observed that the proportion of empty ephippia was lower in lake samples than in sediment traps, which may be explained by the abortion of resting eggs before the ephippium is released, as observed for this species in Río Seco Lake (Conde-Porcuna *et al.*, 2011). The consequent reduction in eggs in the released ephippium would increase the proportion of empty ephippia in the traps.

Various authors have observed that sinking rates are higher for epibiont-infected versus non-infected animals (Herman and Mihursky, 1964; Allen *et al.*, 1993; Barea-Arco *et al.*, 2001). Accordingly, the number of empty ephippia and total ephippia from epibiont-infected animals in the sediment traps should be larger at higher levels of epibiont burden. However, the numbers of total or empty ephippia in the traps were not significantly correlated with epibiont burden in the present study. Furthermore, although empty ephippia weigh less than ephippia with eggs, the proportion of empty ephippia was higher in the sediment traps at low levels of epibiont burden (Fig. 4), which would be attributable to a differential production of the two types of ephippia under these conditions. This finding also indicates the positive effect that epibiont algae may have on ephippial reproduction in *Daphnia*.

Various authors have described the influence of food quality (*Cryptomonas* sp. versus *Scenedesmus obliquus*) on the resting egg production of different *Daphnia* species (Abrusán *et al.*, 2007; Koch *et al.*, 2009), but their conclusions have been inconsistent. It has been proposed that food quantity may reverse the effects of food quality on ephippial production in *Daphnia* (Koch *et al.*, 2009), and the effect of food quality on ephippial production remains unclear. *Korshikoviella gracilipes* appears likely to be a high-quality food, although this aspect has not been studied to date.

The negative relationship observed between *Daphnia* burden and the concentration of TN suggests that epibiont algae may use zooplankton excretion as a nitrogen resource when this element is less abundant (Barea-Arco *et al.*, 2001). In addition, although statistical significance was not reached, there was a trend towards a negative association between lake water DIN:SRP ratios and the proportion of empty ephippia, which may suggest that nitrogen limitation could increase the proportion of empty ephippia. These results may suggest that nutrients may possibly play a role in the ephippial reproduction of *Daphnia*. Smith *et al*. (Smith *et al.*, 2009) found that per capita ephippia production was limited by P when food was abundant but was not significantly affected by food quality under food limitation conditions. They proposed that a combination of food quality/quantity and population density may together induce the production of resting eggs in invasive species. Further research is needed to clarify the possible influence of nutrients on ephippial reproduction.

Kerfoot *et al*. (Kerfoot *et al.*, 2004) reported that the number of empty ephippia detected in sequential traps may be increased by resuspension of sediments with older ephippia. Given that the proportion of empty ephippia among those on the lake bottom is likely higher (due to subsequent hatching events) than the proportion among freshly formed ephippia, a higher proportion of empty ephippia could be expected in the traps under resuspension conditions. However, according to our results, the impact of resuspension appears to have been negligible.

In shallow lakes, the resuspension of bottom materials is mainly driven by wind-induced currents and wave

action (Kerfoot *et al.*, 2004; Kelderman *et al.*, 2012), which are the only relevant mechanisms in the absence of fish. If the material in the sediment traps consisted almost exclusively of resuspended sediment (or ephippia), a positive exponential relationship could be expected between wind speed and sediment yield (Kelderman *et al.*, 2012). In the present study, however, no significant correlation (linear or exponential) was found at any time between daily wind speed, relatively stable during most of the study period, and the number of ephippia or proportion of empty ephippia in the sediment traps.

Nevertheless, not only the average wind speed but also the wind direction and (often short) periods of extreme winds may be equally or even more important (Lick *et al.*, 1994; Schaaff *et al.*, 2006, Kelderman *et al.*, 2012). De Vincente *et al*. (De Vicente *et al.*, 2010) observed that high resuspension fluxes in shallow lakes were related to the maximum wind speeds prior to the sampling date. In the present study, no significant relationship was found between the number of sedimented ephippia and temporal wind direction patterns, and the proportion of empty ephippia in the traps was not significantly associated with the wind speed from the most exposed directions (northeast and southwest), with the maximum wind speed in each period, or with the direction of the wind.

According to these results, wind parameters appear to exert no influence on the ephippia collected in our traps; therefore, a possible resuspension of ephippia does not seem to have influenced our data. In fact, there was no difference in the number of eggs per ephippium between the traps and lake samples. Moreover, the number of ephippia and ephippial eggs found in the traps showed no spatial differences, consistent with our proposition that resuspension by wind was not a relevant factor. De Stasio (De Stasio, 1989) also found that resuspension had only a minor effect on the number of copepod resting eggs collected in sedimentation traps. In the present case, very few ephippia were detected in the lake samples during June, before the onset of ephippia production.

Nevertheless, some of our observations may suggest an influence of this phenomenon in the present study. No ephippial female was collected in the lake during July or August (Fig. 3), but ephippia were collected in the sediment traps over this time (Fig. 5). In addition, the largest quantities of ephippia in sediment traps were observed at the beginning of November, when few ephippial females were found in the lake samples (Figs 3 and 5). However, it should be borne in mind that *Daphnia* densities in the lake were determined from samples obtained on single days, whereas the sediment trap data refer to the number of ephippia accumulated over the intervening time period; therefore, no correlation should necessarily be expected. Hence, there may be two explanations for apparent

mismatches between the absence of ephippial females in the lake (e.g. on 21 and 27 July) and the collection of ephippia in the traps (e.g. on 27 July): (i) a small number of ephippial females may have appeared between these dates (e.g. 21–27 July), and/or (ii) the number of ephippial females was too small for their collection in the lake samples, but the few ephippia produced may have accumulated in the traps over this time period.

Moreover, the distribution of *D. pulicaria* in Río Seco Lake is very patchy (Barea-Arco *et al.*, 2001) and a possible sampling error must be taken into account, although we sampled five random points in a lake of only 0.4 ha in order to minimize this possibility. The *Daphnia* density estimates should be considered with caution; however, they clearly indicate that few ephippia were collected in the traps and adult or ephippial *Daphnia* densities were low during most of the summer, whereas numerous ephippia were found in the traps and adult or ephippial *Daphnia* densities were high at the end of the summer and during the autumn. If ephippial females are more abundant during the autumn, the number of ephippia collected in the traps will also be higher during this season, as observed. Furthermore, the production of empty ephippia recorded in the present investigation is consistent with previous experimental findings that showed a high direct production of empty ephippia and abortion of resting eggs in the same species from the same lake (Conde-Porcuna *et al.*, 2011). Nevertheless, further research is required to rule out in a definitive manner any possible influence of wind resuspension on the number of ephippia collected in the traps.

In summary, despite the considerable research on *Daphnia*, *in situ* ephippial egg production has been poorly studied in the field, especially in obligate parthenogenetic populations and/or in high mountain lakes. According to the present results, ephippia production appears to be related to photoperiod and then, once ephippia are produced, ephippial egg production appears to be associated with food conditions (edible algae biomass and/or epibiont burden). Future research into *Daphnia* and cladoceran reproduction should include studies on the *in situ* production of ephippial eggs (not only ephippia) and the influence of food and nutrient concentrations.

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