On the presence of parthenogenetic populations of *Heterocypris bosniaca* Petkowski et al., 2000 (Crustacea: Ostracoda) in temporary rock pools of the eastern Iberian Peninsula, with insights into the ecology and life history of the species

Josep Antoni Aguilar-Alberola & Francesc Mezquita

Dep. of Microbiology and Ecology, Univ. of València, Av. Dr. Moliner, 50. E-46100 Burjassot (mezquita@uv.es)

Aguilar-Alberola, J. A. & F. Mezquita. On the presence of parthenogenetic populations of *Heterocypris bosniaca* Petkowski et al., 2000 (Crustacea: Ostracoda) in temporary rock pools of the eastern Iberian Peninsula, with insights into the ecology and life history of the species. *Bulletin de la Société des naturalistes luxembourgeois* 109: 149-158.

Abstract. *Heterocypris bosniaca* Petkowski et al. 2000 was abundantly collected from eight temporary rock pools on carbonate substrate near Rafelguaraf (València, Spain). As only females were found, reproduction is inferred to be parthenogenetical. *H. bosniaca* was the only ostracod occurring in the pools investigated. The populations at two sites were weekly monitored from early December 2004 to late January 2005 to investigate the environmental conditions, the population dynamics, larval growth and life history. In parallel, a number of juveniles were reared in the laboratory for controlled instar growth and life-span measurements. The variation in shape throughout the larval development is shown with the help of scanning electron micrographs. The length measurements taken from animals reared separately in the laboratory permitted to better establish size boundaries between the instars in general and in particular in those collected from the field. In the laboratory, the youngest instars stretched over about 50 hours, the last one (A-1) over about 100 hours. Three of the females each laid between 7 and 12 eggs. The species was found to be resistant to desiccation and tolerant to hypoxic conditions.

Key words. Temporary rock pools, population ecology, geographical parthenogenesis, non-marine Ostracoda, València, Iberian Peninsula.

1. Introduction

Heterocypris bosniaca was first described by Petkovski *et al.* (2000) from a well in Bosnia (Balkan peninsula). Both males and females were present. More recently, Martens et al. (2002) reported the finding of three populations, two of them with males, in temporary rock pools in Israel.

In 2004, we first identified the ostracods kept since 1998 in a small aquarium in the laboratory of our institute as belonging to *H. bosniaca*.

The aquarium contained substratum and water taken from a pool located in the mountains of Racó de Rius in Rafelguaraf (València, Spain).

The finding of *H. bosniaca* was the first for the Iberian Peninsula (Aguilar-Alberola & Mezquita 2005), and therefore we decided to investigate the distribution of the species in the area. We also wanted to find out if the absence of males was anecdotic or a constant feature of these populations.

We wanted to find out if the species has built up stable populations in the area or if the populations undergo wide changes that could bring them to local extinction.

We weekly monitored the populations at two sites from the beginning of the rainy period to the complete drying-up of the pools. Also, a number of individuals were reared in the laboratory in order to study the life-cycle and the morphology of the carapace during larval development.

2. Material and methods

2.1. Sampling area

The area of study is situated in the mountains of Racó de Rius in Rafelguaraf, province of València, Spain.

It is covered by typical Thermo-Mediterranean vegetation, composed of pine trees and shrubs. The geological substrate consists of limestone, which through weathering by rainwater forms a high number of small, rounded stony holes that fill up with water during rainy periods. The bottom of the pools is covered mainly with pine needles. The water evaporates relatively quickly so that either in spring, if the season is dry, or otherwise in early summer the habitats have completely dried out.

2.2. Exploratory sampling campaigns

Shortly after the rainy period, we started four sampling campaigns between the 27th of November 2004 and the 18th of December 2004. During these surveys, we searched through flat and deep zones of the area looking for sites with a high probability of holding rain water for several weeks, such as wide stony flat surfaces. We avoided areas with sandy substrate or with a cover of high shrubby vegetation.

At each sampling site, we filled a standardised field form with general information of the pool and surrounding area. This protocol included taking a picture of the pool, and recording GPS coordinates. Size (maximum length and width) and depth at three points were recorded in order to get an approximate idea of the surface (considering they are approximately circular in shape) and volume (most pools have vertical walls and hence a cylindrical structure). *In situ* measurements of water temperature, electric conductivity, pH (HANNA instruments), and oxygen concentration (WTW F/set3 instrument) with field probes were taken. Water samples were collected using plastic jars and transported to the lab in order to analyse chloride concentration and alkalinity using standard volumetric methods (APHA-AWWA-WEF 1992). Ostracods were collected with a small hand net (mesh size 100 µm, diameter 15 cm) swept through the substrate, and fixed in the field with 30% ethanol. In some pools, where densities were very high, the ostracods were collected with a small plastic jar (5 cm in diameter), which was swept smoothly through the muddy substrate.

After several ponds with dense populations of *H. bosniaca* had been found, two of them were selected for the weekly monitoring of the population dynamics and the environmental factors associated to them (see below).

2.3. Weekly monitoring

The two study pools were visited once in the week from the 27th of November 2004 to the 22nd of January 2005, when eventually they both dried up. The two pools, designated as X1 and X6, were selected because of their easy access, high ostracod densities and differences in size and habitat conditions.

On each visit, we measured a number of environmental variables and collected water and ostracod samples. Ostracods were collected with a 100 ml, 5 cm diameter plastic jar, which was introduced into the substrate to a depth of about 1 cm and swept through it over about 3-5 cm. We chose this method because the high densities of *H. bosniaca* in the selected pools allowed collection of high numbers of individuals in this way, and also in order to minimise the impact on these small habitats. The samples collected were fixed with ethanol and later in the lab ostracods were handpicked under a binocular microscope and stored in 70% ethanol. The carapace length of all the individuals collected was measured in order to sort them into size groups that reflected the developmental stages. Adult and A-1 individuals were measured with a precision of 20 µm, A-2 and A-3 with an 10 µm error level, and the rest of instars were measured to the nearest 5 µm.

2.4. Ostracod cultures

In order to obtain precise information on the number, size and duration of the different instars, a few juvenile individuals collected from pool X1 were grown separately at room temperature (20-27 ºC). The cultures were carried out using multi-well

plates filled with filtered water from the pool and dried *Spirulina* sp. as food (Schmit et al. 2007). We monitored each animal individually in its well in order to check moulting events and collect information on the time elapsed between moults and total duration of the larval development.

Daily, sometimes twice per day, each individual was checked for size, valve remains and survival. In order to calculate life span for each instar, we used the between-moults time assessed, or the time to death since last moult in the case of adults. The time since the beginning of the experiment to the first moult is not considered here, so that we do not present information on A-8 life time, because we did not measure time at hatching events. Ostracod valves shed during moulting were collected and measured in order to have a more precise information about the size of each instar.

3. Results

3.1. The rock pools

During the survey *H. bosniaca* was found in eight rock-pools (Table 1). All of them are holes on rocky surfaces and result from the chemical weathering by rainwater. They are surrounded by Mediterranean-type shrubs rooted in neighbouring holes and fissures (Fig. 1). The rockpools being mostly situated on gently sloping land, the intensity of the incoming sunlight mainly depends on the surrounding vegetation. The only exception is pool X3, which is located on a steep slope, but is exposed to low sunlight intensity, this because the water level is about one meter below the rock surface.

The pools differ only slightly in their water chemistry. For instance, pH values ranged between 7.6 and 8.2, and electric conductivity (EC) varied between 150 and 580 µS cm^{-1}, this mainly in relation to pool size, rain events (decreasing EC) and evaporation periods (increase in EC).

Out of these eight sites, the pools X1 and X6 were selected for weekly monitoring. These two sites differ in size (32 x 59 and 92 x 120 cm, respectively), the intensity of direct sunlight, and the fact that X6, receives organic

Table 1. Physical and chemical variables recorded for the eight pools in which *H. bosniaca* was found.

Pool	UTM Coordinates	Date	Temp (°C)	EC (µS cm^{-1})	pH	Cl^- (mg L^{-1})	HCO_3^- (mmol L^{-1})	pO_2 (%)	O_2 (mg L^{-1})	Length (cm)	Width (cm)	Depth (cm)	Area (dm^2)
X1	30S 0721767 4325537	11/12/04	11	180	7.9	14	1.9	75	7.5	59	32	4.6	16
X2	30S 0721754 4325545	27/11/04	16	420	8.2	30	4.1	93	9.5	38	25	7.3	8
X3	30S 0722134 4325146	27/11/04	17	380	7.9	32	3.5	84	9.2	51	37	8.1	15
X4	30S 0722158 4325260	27/11/04	14	350	7.9	30	4.5	88	10.0	60	42	13.2	20
X5	30S 0722158 4325259	27/11/04	13	370	7.6	31	3.8	85	11.0	75	57	5.7	34
X6	30S 0721884 4325380	11/12/04	14	160	8.0	8	1.9	93	8.8	120	92	28.1	88
X7	30S 0721887 4325367	11/12/04	14	160	8.2	10	1.9	111	11.0	60	41	5.1	20
X8	30S 0721896 4325365	18/12/04	13	150	7.7	n.a.	2.1	91	12.0	56	48	7.7	21

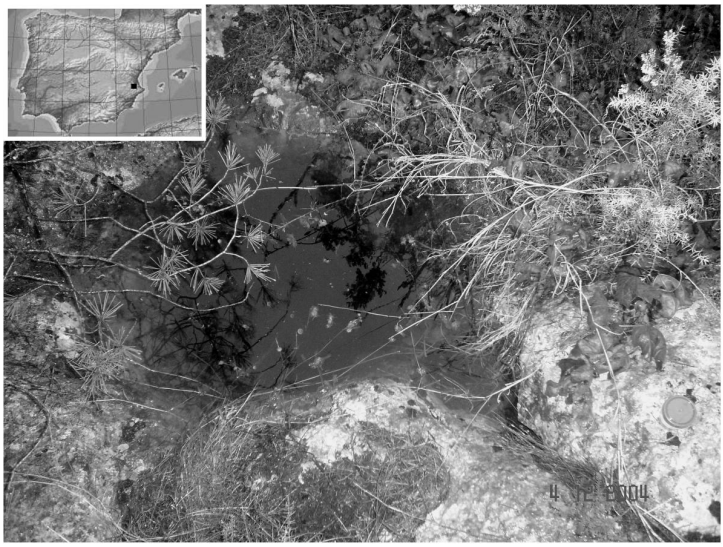


Fig. 1. Site X6, where its general shape can be seen, so as the type of rocky substrate and surrounding shrub vegetation rooting on surrounding holes and crevices.

matter not only from pine needles but also from fig tree leaves (*Ficus carica*) in autumn. It also harbours a population of *Branchipus schaefferi*, an anostracan which in lab cultures we saw to be subjected to predation by *H. bosniaca*.

In general, pool X1 shows greater fluctuations of the environmental variables than X6 (Fig. 2). During the study, conductivity increased gradually in X6 whereas in X1 large fluctuations were recorded. The oxygen concentration was less variable in X1 (between 5.5 and 11 mg L^{-1}) than in X6, where a minimum of 2,4 mg L^{-1} was measured.

3.2. Sex, size and carapace shape of the instars

Heterocypris bosniaca was found to be the only ostracod occurring in the eight pools. All the individuals collected were females, so far no males have been found in the pools studied. From this finding we would like to infer that reproduction in the pools is exclusively parthenogenetical.

The A-8 instars have a rounded quadrate shape with an almost straight dorsal margin when seen laterally (Fig. 3). The oblong carapace shape of the adults is progressively reached throughout the larval stages.

Table 2. Average, median, minimum and maximum carapace length, number of individuals (N) measured for each instar, and moult increment for the median length of a given instar compared to the previous one.

Instar	N	Maximum (mm)	Minimum (mm)	Mean (mm)	Median (mm)	Moult increment % (median)
A	432	1.50	1.22	1.37	1.38	35.3
A-1	997	1.20	0.94	1.05	1.02	34.2
A-2	1153	0.90	0.67	0.77	0.76	31.0
A-3	606	0.70	0.50	0.58	0.58	26.1
A-4	561	0.51	0.40	0.46	0.46	24.3
A-5	189	0.40	0.33	0.37	0.37	27.6
A-6	88	0.33	0.25	0.28	0.29	16.0
A-7	4	0.26	0.21	0.24	0.25	13.6
A-8	2	0.23	0.21	0.22	0.22	15.8
EGG	5	0.19	0.18	0.19	0.19	-

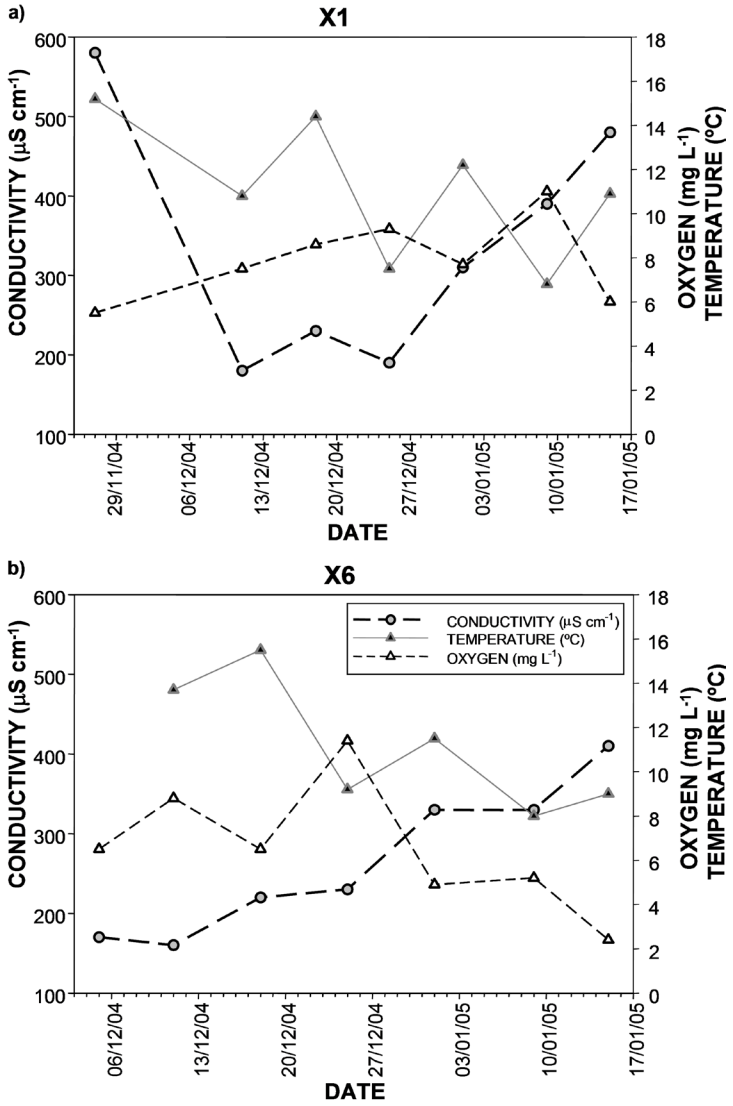


Fig. 2. Graphs showing temporal changes in water temperature, electrical conductivity and oxygen concentration in pools X1 (a) and X6 (b) during the study period.

The increase in size between two moults in general increases throughout larval development (Table 2). Consequently late instars are more readily separated from each other under the microscope than smaller ones.

This can also be seen if we plot the size (carapace length) frequency distribution for all individuals measured in pool X6. Several modes clearly correspond to late (older) instars. By contrast, juvenile bell-shaped distributions clearly overlap (Fig. 4). Despite the low number of individuals used in our growth experiments, the sizes obtained from lab-animals with known instar belonging allowed us to better establish size boundaries of the instars in general (Fig. 4).

Upon comparing the carapace length of the individuals collected from pool X1 with those collected from X6, it can be seen that median length is always higher for those collected from X6 (Fig. 5).

3.3. Population dynamics

Throughout the eight weeks of the study period (late November 2004 to late January

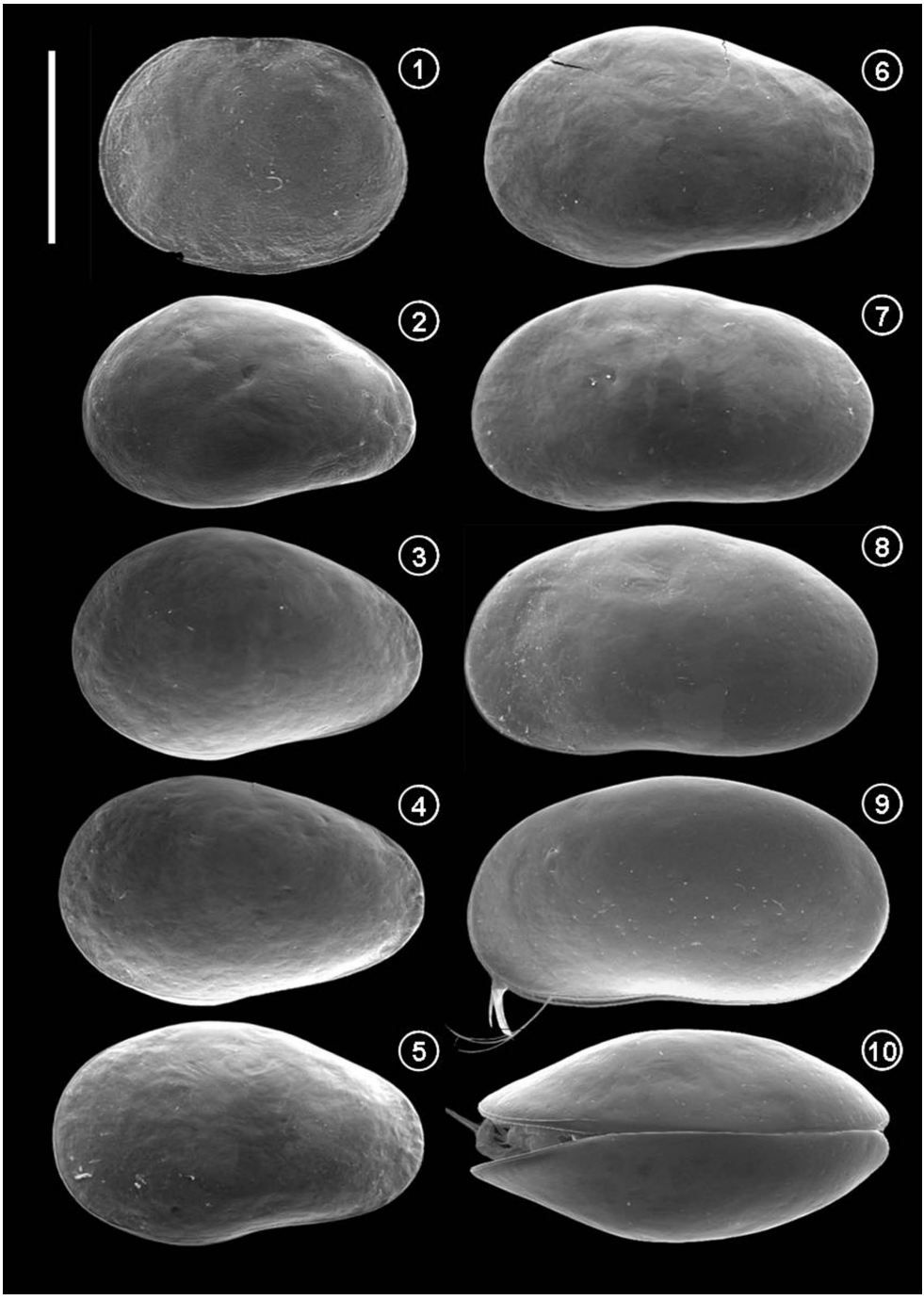


Fig. 3. External views of different instars of *H. bosniaca*. (1): A-8, left valve. Scale bar = 132 µm, (2): A-7, left valve. Scale bar = 139 µm, (3): A-6, left valve. Scale bar = 155 µm, (4): A-5, left valve. Scale bar = 197 µm, (5): A-4, left valve. Scale bar = 240 µm, (6): A-3, left valve. Scale bar = 290 µm, (7): A-2, left valve. Scale bar = 371 µm, (8): A-1, left valve. Scale bar = 495 µm, (9): Adult female, left valve. Scale bar = 632 µm, (10): Adult female, dorsal view. Scale bar = 632 µm.

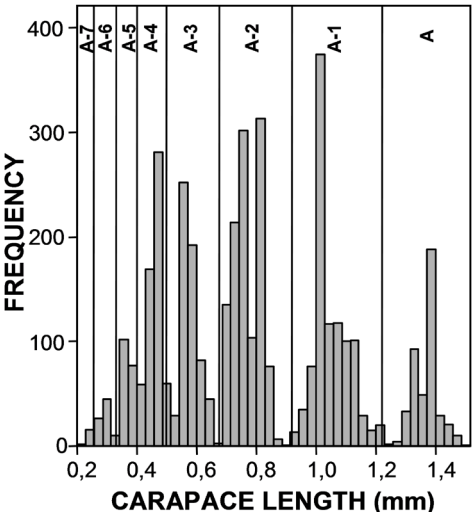


Fig. 4. Size frequency histogram showing the distribution of carapace lengths (CL) for pool X6. Vertical lines show the CL boundaries for the different instars, as obtained from laboratory cultures.

2005) there was a continuous increase in the proportion of older instars in both pools (L1 and L6). Additionally, a slight increase in the number of early juveniles was seen in pool X6 at the middle of the study period (Fig. 6).

3.4. Instar life spans

During the experimental cultures, the periodic checking of individuals allowed us to determine to some extent the time elapsed between two consecutive moulting events for each animal under study. In this way, we could calculate the life span of each growth instar and, consequently, of the whole life-cycle of an individual (excluding egg and A-8 life time) (Fig. 7). In general, the youngest juvenile instars lasted for about 50 hours each, whereas the oldest juvenile instar (A-1) lived for about 100 hours before moulting to adulthood. Adult individuals lived longer than 250 hours under the lab conditions used in our experiments. Three of the females that had reached sexual maturity each laid between 7 and 12 eggs.

4. Discussion and conclusion

The eight pools in which *H. bosniaca* was found in Rafelguaraf were very similar in their habitat conditions. The environmental factors analysed, in spite of varying within a rather narrow range only, present fluctuations that are largely a result of the seasonality of the water body, for instance conductivity decreases after rainy periods to increase towards the end of the hydroperiod.

The high densities of *Heterocypris bosniaca* in this type of environment, together with the fact that this was the only ostracod present, clearly indicate that the species is tolerant to desiccation and fluctuating and unpredictable environments.

From the fact that the nine sampling sites are all distributed over a 9 ha area, with a few of them being quite isolated from the others, *H. bosniaca* appears to have a good dispersal ability, at least at the small local scale.

Furthermore, the low oxygen concentrations observed at particular dates (e.g., c. 2mg L^{-1}

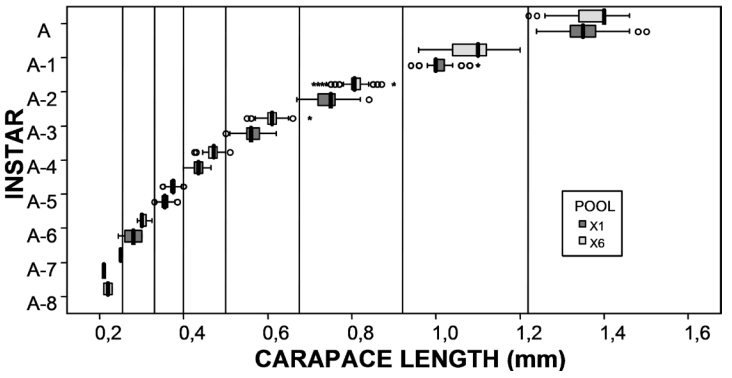


Fig. 5. Boxplots showing the carapace length (CL) distribution for the different instars, separately for pools X1 and X6. Vertical lines show CL boundaries obtained from laboratory cultures for each juvenile instar.

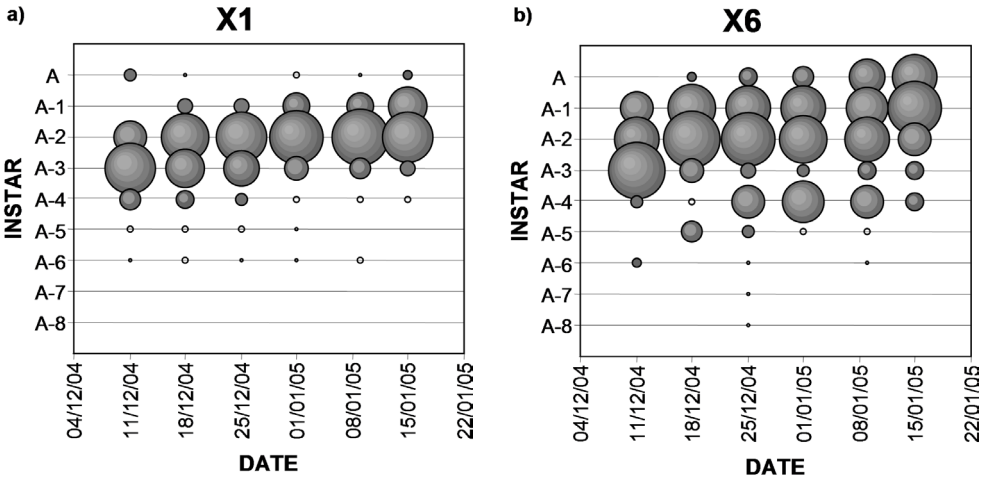


Fig. 6. Temporal changes in relative abundances of different instars of *H. bosniaca* in pools X1 (a) and X6 (b). Bubble size is related to percentage of individuals of a given instar in the sample.

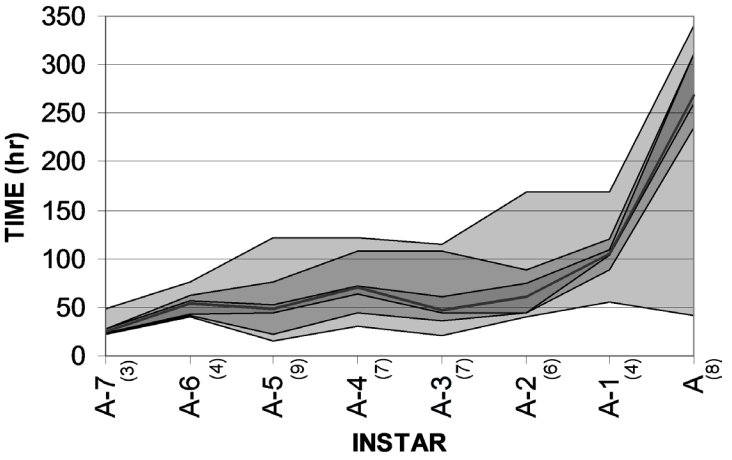


Fig. 7. Life span for each instar of *H. bosniaca* as calculated from laboratory experiments. The figure shows: i) minimum and maximum time span per instar (pale grey area), ii) time span range excluding outliers (intermediate grey area), iii) median time span per instar, including measuring error (dark grey area) and iv) median time span per instar (dark grey line). Number of measurements between brackets.

at the end of the wet season in X6) indicates that the species is tolerant to hypoxia, a trait known from other members of the genus, so for instance *H. incongruens* (Margalef 1953).

Similarly to other ostracod species (*e.g.* Smith & Martens 2000), *H. bosniaca* presents moult increments that are progressively larger from early juvenile carapace length to adult size (increasing approximately at each moult from 14 to 35 %).

The differences in carapace length between individuals of the same instar living in X1 *versus* X6 are most likely attributable to the environmental differences between these two pools; environmental variables fluctuate less abruptly in X6, it contains a higher amount of organic matter derived from fig tree leaves and, in addition, harbours a population of anostracans that could serve as food to the ostracods. Thus the higher amount of food resources in X6 might explain the larger mean size of the ostracods it harbours.

The fact that the presence of the species in some of the studied pools can be dated back to ten years ago, when the first animals were collected and stored in a personal aquarium, suggests that *H. bosniaca* has been living in such temporary water bodies with success

thanks to the high tolerance of its diapausing eggs to desiccation, because the pools dry up in summer for several months, and in some dry years they must have a very short hydroperiod. The appearance of a second cohort in X6 during the study period also suggests that the species is able to produce subitaneous eggs, in a similar way as its congener *H. incongruens* (Angell & Hancock, 1989).

The experimental results show that *H. bosniaca* has a relatively short life-cycle, as it takes A-7 juveniles only about 17 days to reach adulthood at temperatures above 20°C. Latifa (1987) obtained similar results for *H. incongruens*: larval development stretched over 16.7 days at 20°C and 12.5 days at 25°C, respectively.

The largest difference observed between the two species relates to the life-span of A-1 juveniles. In *H. bosniaca* this instar lives almost twice as long as the previous instar, whereas in *H. incongruens* A-1 juveniles show a life-span similar to the earlier juvenile instars (Latifa 1987). The latter author (op. cit.) also reported a clear shortening of the larval development with increasing temperatures. We did not check for this effect in the laboratory, but our field data indicate that the animals were taking longer from one moult to the next than under lab conditions. This is most likely due to the higher temperatures in the lab (20-27°C) compared to those measured in the pools (17°C).

The absence of males in the study area is not surprising, as geographical parthenogenesis is a somewhat frequent pattern in cypridid ostracods, and in the genus *Heterocypris* in particular (Horne et al. 1998).

The finding of high density populations of *H. bosniaca* demonstrates that the species has established stable populations in the area studied. The species is highly tolerant to desiccation, most probably through the production of resting eggs, and it is probably able to lay subitaneous eggs too. It seems also tolerant to some degree of fluctuations in water ionic and oxygen concentrations.

Parthenogenetic reproduction may explain long-distance dispersal in *H. bosniaca*. Indeed one single ovigerous female or even one single egg can give rise to a new population or even metapopulation as the one observed in the area investigated.

Further studies are needed to understand the geographic origin and genetic structure of these populations.

Acknowledgements

We are grateful to Dietmar Keyser (University of Hamburg) for confirming the identity of the species. This work was partly funded by the EU Marie Curie Research Training Network SexAsex (From Sex to Asex: a case study on interactions between sexual and asexual reproduction, contract MRTN-CT-2004-512492). Claude Meisch (National Natural History Museum of Luxembourg) is greatly acknowledged for his help in reviewing the manuscript and suggesting improvements.

References

Aguilar-Alberola, J. A. & F. Mezquita, 2005. Population dynamics of *Heterocypris* cf. *bosniaca* in temporary rock pools in València (Spain). In: Kohring, R. & B. Sames, (eds.), 15th International Symposium on Ostracoda, Berlin, September 12-15, 2005, *Berliner Paläobiologische Abhandlungen, Band* 6: 9.

APHA-AWWA-WEF. 1992. Standard methods for the examination of water and wastewater. 18th edition. American Public Health Association. Washington D.C.

Angell, R. W. & J. W. Hancock, 1989. Response of eggs of *Heterocypris incongruens* (Ostracoda) to *experimental* stress. *Journal of Crustacean Biology* 9 (3): 381-386.

Horne, D. J., A. Baltanás, & G. Paris, 1998. Geographical distribution of reproductive modes in living non-marine ostracods, In: Martens, K. (eds.), *Sex and parthenogenesis: evolutionary ecology of reproductive modes in non-marine ostracods* 77–99; Backhuys, Leiden, the Netherlands.

Latifa, G. A., 1987. Effect of temperature on the development of embryonic and postembryonic stages of *Heterocypris incongruens* (RAMDOHR, 1808) (Ostracoda). *Crustaceana* 52(1): 95-100.

Margalef, R., 1953. Los crustáceos de las aguas continentales ibéricas. Inst. For. Invest. Exp., Madrid.

Martens, K., S. S. Schwartz, C. Meisch & L. Blaustein, 2002. Non-marine Ostracoda (Crustacea) of Mount Carmel (Israel), with

taxonomic notes on Eucypridinae and circum-Mediterranean *Heterocypris*. *Israel Journal of Zoology* 48: 53-70.

Petkovski, T., B. Scharf & D. Keyser, 2000. New and little known ostracods of the genus *Heterocypris* (Crustacea, Ostracoda) from the Balkan Peninsula. *Limnologica* 30: 45-57.

Schmit, O., G. Rossetti, J. Vandekerkhove & F. Mezquita, 2007. Food selection in *Eucypris virens* (Crustacea: Ostracoda) under experimental conditions. *Hydrobiologia* 585: 135-140.

Smith, R. J. & K. Martens, 2000. The ontogeny of *Eucypris virens* (Cyprididae, Ostracoda). *Hydrobiologia* 419: 31-63.