Ann. Zool. Fennici 51: 433–444 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 31 October 2014

Endogenous seasonal variation in the encapsulation response of the noble crayfish (*Astacus astacus*)

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Received 27 Nov. 2013, final version received 25 Mar. 2014, accepted 11 Apr. 2014

Gruber, C., Vainikka, A., Hirvonen, H., Rantala, M. J. & Kortet, R. 2014: Endogenous seasonal variation in the encapsulation response of the noble crayfish (*Astacus astacus*). — *Ann. Zool. Fennici* 51: 433–444.

Seasonality in the immune defence of invertebrates can coincide with environmental variation but whether it is endogenously regulated, via biological clocks, or affected by previous immune challenges remains unclear. Using the native noble crayfish (*Astacus astacus*) held under constant laboratory conditions for a year, we explored (1) potential endogenous seasonal variation in immune defence, i.e. the encapsulation response, (2) the potential positive effect of repeated challenges with a standardized immune insult in subsequent seasons, i.e. long-lasting immune priming, and (3) whether long-lasting immune priming is dependent on endogenous seasonality. Independent measurements of the encapsulation response in different seasons revealed significant variation and a decrease in autumn. This result indicates previously undetected endogenous seasonal variation in invertebrate immunity. The weaker immune defence observed in autumn, i.e. the reproductive season of crayfish, might be caused by a circannual clock. When corrected for endogenous seasonality, we found no evidence for long-lasting immune priming.

Introduction

Immune defences have evolved to minimize the fitness costs of infections by controlling them (Schmid-Hempel 2011). A major assumption in ecological immunology, arising directly from life-history theory, is that immune defences are costly to produce, maintain and activate, and cannot be maximised due to energetic and nutritional constraints (Schmid-Hempel 2011). Empirical evidence shows that immune defences are not only affected by host genotype (vertebrates: Trowsdale & Parham 2004, invertebrates: Lazzaro *et al*. 2004) and evolution (vertebrates: Best & Kerr 2000, invertebrates: Pauwels *et al*. 2010) but also by the features of the individual, such as condition, reproductive state and sex (Schmid-Hempel 2011). Recently, temporal variation in the immune defence of vertebrates and invertebrates has received growing interest (Kortet & Vainikka 2008, Martin *et al*. 2008, Hawley & Altizer 2011), especially with regard

to the underlying mechanisms (Lee & Ederyl 2008, Watthanasurorot *et al*. 2011, Noonin *et al*. 2013). Another topic of increasing interest is the protective effects of a repeated exposure to parasites and pathogens, i.e. immune priming in invertebrates (Schmid-Hempel 2011).

Temporal variation in immune defence is of interest because it potentially indicates differences in host susceptibility to and survival from infections over time (Kortet & Vainikka 2008, Hawley & Altizer 2011). Fluctuations in environmental cues, such as photoperiod, temperature, food availability or pathogen abundance, may induce temporal variation in immunity directly (Nelson *et al*. 2002, Kortet & Vainikka 2008) or indirectly due to energetic tradeoffs between immune defence and competing behavioural and physiological activities (Kortet & Vainikka 2008, Martin *et al*. 2008), which depend on environmental cues. However, some empirical studies in vertebrates and invertebrates also demonstrate temporal variation in immune defence when environmental cues are kept constant (Brock 1983, Lee & Ederyl 2008). This endogenous variation is likely to be genetically controlled through biological clocks (Sharma 2003, Paul *et al*. 2008, Wikelski *et al*. 2008). Biological clocks have evolved in response to the periodicity in the environment, and they time the behaviour and physiology of an individual, even in the absence of environmental cues (Sharma 2003, Paul *et al*. 2008, Wikelski *et al*. 2008). Recently, an increasing number of studies have suggested that circadian (24 h periodicity) clocks influence immune defences in vertebrates (Keller *et al*. 2009) and invertebrates (Lee & Ederyl 2008, Watthanasurorot *et al*. 2011, Stone *et al*. 2012, Noonin *et al*. 2013). However, so far only empirical studies in vertebrates indicate an endogenous regulation of seasonality in immune defence (Brock 1983, Kiank *et al*. 2007). Their results suggest a seasonal (circannual) rhythm in immune defence that is probably generated by a circannual clock, since the observed pattern reoccurred under constant environmental conditions in approximately 12-month cycles (Paul *et al*. 2008). In invertebrates, the endogenous regulation of immunity at a seasonal scale has received little attention due to the generally short lifespan of most invertebrates and, consequently, the inherent difficulty in building experimental setups at this temporal scale. Nevertheless, seasonal variation in the immune defence of invertebrates is observed in the wild (Kortet & Vainikka 2008, Dissanayake *et al*. 2011) and can coincide with the seasonal pattern of photoperiod and temperature (e.g. crustacean studies reviewed in Le Moullac & Haffner 2000, Mydlarz *et al*. 2006). Therefore, our study aimed to investigate the potential existence of endogenous seasonal variation in the immune defence of invertebrates, here represented by crayfish.

In addition to a potential endogenous regulation, varying and previous exposures to parasites and pathogens can cause temporal variation in the immune defence of invertebrates. For example, Krams *et al*. (2013) reported that a previous immune insult, i.e. a challenge with a nylon monofilament implant one week prior, enhanced the strength of immune defence, measured as the encapsulation response, to a subsequent challenge with an implant and increased survival from a fungal infection in a beetle species. This enhanced immune defence to subsequent challenges in invertebrates is defined as immune priming (Schmid-Hempel 2005, Rodrigues *et al*. 2010). Immune priming can be long-lasting (Moret & Siva-Jothy 2003, Schmid-Hempel 2005, Sadd & Schmid-Hempel 2006, Roth & Kurtz 2009, Rodrigues *et al*. 2010) and remain for a lifetime in species with a short lifespan, such as *Drosophila melanogaster* (Pham *et al*. 2007). Furthermore, immune priming can be passed to offspring, potentially due to epigenetic effects on immunity (trans-generational immune priming: Sadd & Schmid-Hempel 2007, Tidbury *et al*. 2011). Therefore, any seasonal variation in immune defence could potentially be explained by immune priming. Since recent findings suggest immune priming in crustaceans (Cerenius *et al*. 2003, Kurtz & Franz 2003, Roth & Kurtz 2009, Pope *et al*. 2011, McTaggart *et al*. 2012), we examined whether an encountered immune challenge in a previous season can actually enhance the immune defence of the noble crayfish, *Astacus astacus*, to a repeated challenge in a subsequent season.

For several reasons, the noble crayfish is an ideal model organism to study factors influencing seasonal variation in immune defence.

Immunological research on this and other crayfish species has in general drawn considerable attention (Söderhäll *et al*. 1996, Cerenius *et al*. 2003, Vazquez *et al*. 2009), especially because the noble crayfish is facing a high risk of extinction in the wild (Red List Category & Criteria: Vulnerable A2, IUCN Red List of threatened species) mainly due to the invasive crayfish plague. The crayfish plague, caused by the oomycete *Aphanomyces astaci*, can infect many crayfish species and is usually lethal to European species (Unestam 1972, Jussila *et al*. 2014; *see* also Aydin *et al*. 2014). In Finland, the native noble crayfish has suffered due to the invasive signal crayfish and the associated crayfish plague (Westman *et al*. 2002). Furthermore, the distribution of the noble crayfish extends to the north of Europe and has its northern edge in Finland (Skurdal *et al*. 1999, Souty-Grosset *et al*. 2006), where environmental cues and the life history of crayfish largely differ between seasons. Investigating potential endogenous seasonal variation in their immune defence will hence not only contribute to the general understanding of the mechanisms driving temporal variation but also indicate potential adaptations to strong seasonality in the environment. Furthermore, both endogenous seasonal variation and the effects of previously encountered immune challenges might have implications for the survival of noble crayfish from diseases, such as the crayfish plague.

There were three main aims of this study. First, we aimed to explore whether noble crayfish display endogenous seasonal variation in immune defence. For this purpose, we measured the strength of the encapsulation response to a novel, standardized immune insult, i.e. a nylon monofilament implant (Rantala & Roff 2007), under stable laboratory conditions during a single year. Second, we examined the effect of an immune insult in a previous season on the strength of the encapsulation response to a secondary immune insult in the subsequent season, when the noble crayfish were held in a stable environment. Third, we tested whether a potential stronger encapsulation response to the secondary immune insult in a subsequent season, i.e. long-lasting immune priming, would be dependent on endogenous seasonality.

Material and methods

Study animals

On 3 August 2008, we obtained 172 two- to three-year-old noble crayfish from a commercial crayfish producer in southern Finland. The farmed population had been cultured outdoors, in a predator-free environment, for several generations. Their ancestors originated from a large number of wild noble crayfish specimens from southern Finland. We included 113 crayfish [60 females, body mass 7.28 ± 3.40 g (mean ± SD), and 53 males, body mass 8.23 ± 4.20 g (mean ± SD)] in the experiments and the analyses (Table 1). We collected the data for this study at the Experimental Unit of the University of Oulu between August 2008 and September 2009. The number of crayfish decreased due to mortality before the primary immune challenge (in total 26 individuals) and due to the loss of five implants. Furthermore, during the course of the experiments, 27 crayfish moulted once and one crayfish twice. In total, 19 moultings occurred between 8 September and 25 October 2008 and

Table 1. Details on the noble crayfish (*Astacus astacus*) and the experimental setup of this study.

Group	Primary immune challenge				Secondary immune challenge			
	Sampling time	*n*	Mean mass (g)	Males (%)	Sampling time	*n*	Mean mass (g)	Males (%)
1	13 Oct. 2008	35	9.2	48.6	10 Feb. 2009	33	9.1	48.5
2	10 Feb. 2009	29	6.9	44.8	18 May 2009	23	7.5	52.2
3	18 May 2009	24	7.1	45.8	27 July 2009	15	8.7	46.7
4	27 July 2009	25	7.2	48.0	1 Sep. 2009	14	7.4	42.3

ten between 30 June and 9 August 2009. The moulting cycle is known to affect the immune defence of crustaceans (Le Moullac *et al*. 1997, Cheng & Chen 2001, Cheng *et al*. 2003), potentially due to hormonal changes and energetic trade-offs (Martin *et al*. 2008, Schmid-Hempel 2011). In order to omit the effect of moulting on the strength of the encapsulation response, we excluded the moulted crayfish from the study. Nevertheless, separate analysis showed that including those individuals would not alter our results qualitatively.

Laboratory acclimatization

Upon arrival in Oulu, we individually marked the crayfish for identification using a white marker pen (Textmark 250). We held the crayfish individually in compartments, 105 mm (width) × 145 mm (length) × 230 mm (water depth), built in seven 300-l tanks, which were equipped with a water flow-through system. We haphazardly allocated the crayfish to the tanks and compartments. The individuals in a tank were physically but not chemically separated from each other. We provided each crayfish with a grey plastic tube (75 mm length, 36 mm in inner diameter) for shelter and with *ad libitum* food (carrot, alder leaves and periodically fresh fish and shrimps). In order to reveal endogenous seasonal variation in immune defence, both the light–dark rhythm by artificial lighting (light period from 07:00 to 17:00) and the water temperature (10 ± 1 °C) were kept constant throughout the experiment. We kept the crayfish under these conditions for two months to acclimatise their physiology, before starting the experiments.

Experimental setup

As in other invertebrates, encapsulation is one of the most important nonspecific cellular defence mechanisms in crayfish, whereby a sheath of melanin restricts the growth of an invader and eventually kills it (Cerenius *et al*. 2003, Vazquez *et al*. 2009). In order to study endogenous seasonal variation in immune defence as well as potential long-lasting immune priming, we employed the nylon monofilament implant method (Rantala & Kortet 2004, Rantala & Roff 2007) to induce and quantify the encapsulation response. This method is an easy and informative way to obtain a standardized measure of the strength of the encapsulation response in terrestrial and aquatic arthropods (Smilanich *et al*. 2011, Ardia *et al*. 2012, Dubovskiy *et al*. 2013, Gruber *et al*. 2014). Furthermore, the strength of the encapsulation response to the implant is also strongly related to the defence against parasites (Rantala & Roff 2007, Smilanich *et al*. 2009). We prepared the nylon monofilament (Stroft GTM, Germany, 0.20 mm in diameter) implant, as previously described (Gruber *et al*. 2014), by roughening and knotting the line before cutting it into 6-mm-long pieces. To ensure sterility, we stored the implants in 95% ethanol until they were used.

Due to a lack of information about the social interactions and exposure to pathogens in the past, the individual crayfish may have differed in their immune challenge history. However, we randomly divided the crayfish into four groups with equal sex ratios. To investigate endogenous seasonal variation in the strength of the encapsulation response, the primary immune challenge with an implant of the four groups occurred at different time points (seasons) within a one-year period, while laboratory conditions were kept stable. Due to the limited amount of crayfish available, we assessed the strength of the encapsulation response in specific months that best represent the different seasons in Finland and strongly differ in environmental cues (autumn, winter, spring and summer in Finland; Table 1). More specifically, we chose mid-October (autumn) because natural waters are becoming colder and mature crayfish have already spawned. Early February (winter), on the other hand, is usually the coldest period of the year. In mid-May (spring) lakes have lost their ice-cover and waters have warmed. In late July (summer) natural waters are usually at their warmest. In order to examine potential long-lasting immune priming, we repeatedly challenged the different groups with the standardized immune insult in the subsequent season (two to four months time lag between the challenges; Table 1). We implanted the crayfish of the first group through

a small puncture, pierced with a sterile needle, in the first joint of the left cheliped on 13 October 2008 (autumn). We removed the implants after 48 hours and kept them frozen at –20 °C until analysis. On 10 February 2009 (winter), we repeatedly sampled the encapsulation response of the first group according to the method described above (but from the right cheliped). In addition, the crayfish of the second group were primary challenged. On 18 May 2009 (spring), additionally to the repeated challenge of the crayfish of the second group, we assessed the encapsulation response of the third group for the first time. On 27 July (summer), we implanted the crayfish of the fourth group for the first time additionally to the repeated sampling of the third group. We assessed the encapsulation response of the fourth group for the second time on 1 September 2009. We performed the implantations between 11:00 and 19:00 and always removed the implants after 48 hours. After removing the implants, we always measured the wet body mass (to the nearest 0.1 g) of all individuals.

In order to quantify the strength of the encapsulation response, we photographed each implant from three different angles using a light microscope and an attached digital camera. We analysed the pictures using the ImageJ program (ver. 1.43u, http://rsbweb.nih.gov/ij/) to determine the grey values of reflecting light. Encapsulation response was determined, as previously described (Rantala & Kortet 2004), by subtracting the mean of the three grey value measures from the grey value of a clear implant (having grey value 236).

Statistical analyses

The normality and homoscedasticity of the studied parameters were visually inspected using histograms, normal quantile-quantile (Q-Q) plots and boxplots. Additionally, normality and homoscedasticity were tested using Shapiro-Wilk's and Levene's tests. We log-transformed body mass and square root-transformed encapsulation response in order to meet the assumptions of normality and homogeneity of variance. We allowed the violation of the statistical homogeneity of variances in the encapsulation responses, since visual inspection revealed that the ratio between the largest and smallest variance was below four (Fox 2002).

To investigate differences in the mean encapsulation response to the primary challenge with an implant among seasons, we used an analysis of covariance (ANCOVA) after confirming that the homogeneity of slopes assumption was fulfilled (Engqvist 2005). We ran the full model with season, sex and their interaction as fixed factors, and body mass, measured at the time of the primary immune challenges, as a covariate. Non-significant model terms were stepwise removed. We employed pairwise comparisons among estimated marginal means, using least significant difference (LSD) adjustment, to investigate differences in the strength of encapsulation response among seasons. We also ran a generalised linear mixed model (GLMM) with season and sex as fixed factors, body mass as a covariate and tank as a random factor, to examine possible tank effects. Additionally, using ANCOVA with season and mortality between the primary and secondary immune challenge as fixed factors, and body mass as a covariate, we compared the strength of the encapsulation response between the crayfish that died or survived.

In order to examine potential long-lasting immune priming, we investigated the change in the strength of the encapsulation response between the primary and secondary immune challenge of the same individuals among different seasons using a repeated measures analysis of covariance (RM-ANCOVA). We used the encapsulation responses at the two sampling occasions (primary and secondary immune challenge, repeated measure) as the dependent variable, and season, sex and their interaction as fixed factors. We used body mass, measured at the same time as the primary immune challenges, as a covariate in RM-ANCOVA. Non-significant model terms were stepwise removed. Additionally, we employed pairwise comparisons among estimated marginal means, using LSD adjustment, to investigate differences in the change of the encapsulation response among seasons.

Since we found seasonal variation in the strength of the encapsulation response, we tested whether potential long-lasting immune priming

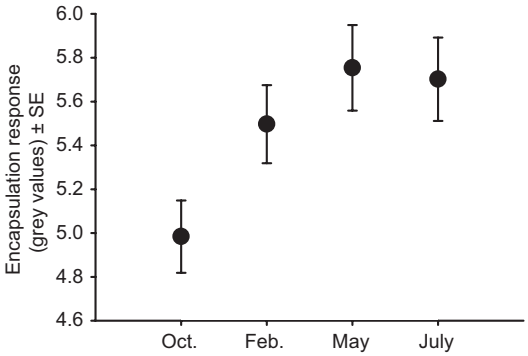

Fig. 1. Endogenous seasonal variation in immune defence. Seasonal variation in the strength of the encapsulation response to the primary immune challenge with a nylon monofilament in the noble crayfish, which were held under stable laboratory conditions (estimated marginal means ± SE from ANCOVA). Encapsulation response was determined, as previously described by subtracting the mean of the implant grey value measures from the grey value of a clear implant (*see* Material and methods).

is dependent on endogenous seasonality or not. We corrected for the seasonality effect by comparing the strength of the encapsulation response between pre-challenged (primary immune challenge in the previous season) and naïve individuals at the same sampling time. Independent individuals were challenged for the first or second time in February, May and July. We were therefore able to examine the effect of a pre-challenge with an implant on the strength of the encapsulation response to a subsequent challenge for those seasons. We ran separate ANCOVAs, with the treatment (naïve or pre-challenged) as a fixed factors and body mass, measured at the time of the primary immune challenges, as a covariate.

Table 2. Endogenous seasonal variation in immune defence. Results of ANCOVA for testing the effects of season and sex on the strength of the encapsulation response to the primary immune challenge with a nylon monofilament in the noble crayfish, using body mass as a covariate. Partial eta-squared (η^2) is reported as an estimate of effect size.

Source of variation	*F*	df	*p*	η^2
Intercept	125.040	1,107	< 0.001	0.539
Season	3.991	3,107	0.010	0.101
Sex	3.783	1,107	0.054	0.034
Log(body mass)	5.923	1,107	0.017	0.052

Results

Endogenous seasonal variation in immune defence

Variation in the mean strength of the encapsulation response varied significantly among seasons (Table 2). According to pairwise comparisons among estimated marginal means, the mean encapsulation response of noble crayfish to the primary immune challenge with an implant was weaker in October as compared with that in February ($p = 0.039$), May ($p = 0.004$) and July ($p = 0.005$) (Fig. 1 and Table 1). Variation in the mean strength of the encapsulation response also tended to differ between the sexes (Table 2). Females (5.659 ± 0.124) had, on average, a stronger encapsulation response than males (5.309 ± 0.131, estimated marginal means ± SE). There was no significant interaction between season and sex ($F_{3,104} = 1.788$, $p = 0.154$, $\eta^2 = 0.049$). The homogeneity of slopes assumption in order to run the ANCOVA was fulfilled, since no interactions between the fixed factors and body mass were significant ($p \geq 0.192$).

The strength of the encapsulation response did not differ between the seven tanks (GLMM: Wald $Z = 0.390$, $p = 0.696$). In total, 28 crayfish died between the primary and secondary immune challenges (Table 1). However, the strength of the encapsulation response after the primary immune challenge did not differ between the crayfish that survived or died (ANOVA: $F_{1,107} = 0.060$, $p = 0.807$, $\eta^2 = 0.001$).

Long-lasting immune priming

The change in the strength of the encapsulation response between the primary and secondary immune challenges was season dependent (Fig. 2 and Table 3). Pairwise comparisons among estimated marginal means revealed that the mean encapsulation response of crayfish first challenged in October significantly increased when challenged a second time in February ($p = 0.003$), whereas the mean encapsulation response of crayfish first challenged in July significantly decreased when challenged a second time in September ($p = 0.009$) (Fig. 2). We

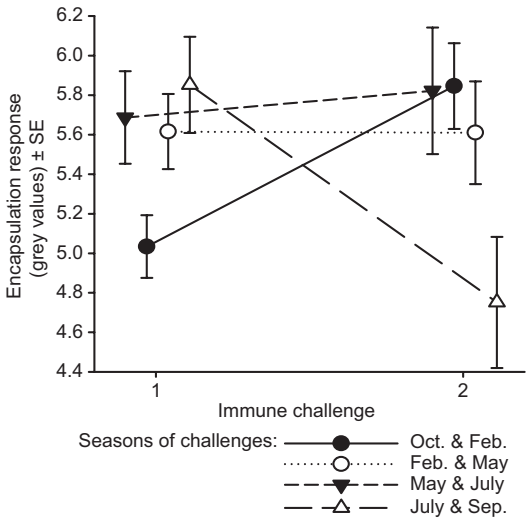

Fig. 2. Long-lasting immune priming. Changes in the strength of the encapsulation response between the primary (1) and secondary (2) immune challenges during different seasons (estimated marginal means ± SE from the RM-ANCOVA).

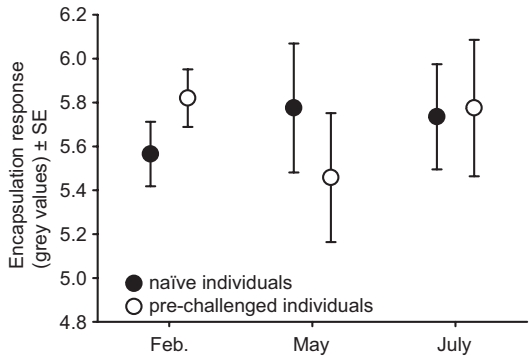

Fig. 3. Long-lasting immune priming corrected for seasonality. The effect of a pre-challenge with a nylon monofilament on the strength of the encapsulation response (estimated marginal means ± SE from separate ANCOVAs). Time lag between primary and secondary challenges of pre-challenged individuals ≥ 2 months (Feb: 4 months; May: 3 months, July: 2 months).

dropped the non-significant factor sex and its interaction with season (within- and between-subject effects $p \geq 0.331$) from the model. The homogeneity of slopes assumption was fulfilled ($p \geq 0.181$).

Long-lasting immune priming corrected for seasonality

The results of separate ANCOVAs for February, May and July revealed that crayfish that were pre-challenged with an implant in the previous season and naïve crayfish that had never been exposed to the implants did not differ in the strength of their encapsulation response to a nylon monofilament implant (Feb.: $F_{1,62} = 1.596$, $p = 0.211$, $\eta^2 = 0.025$; May: $F_{1,60} = 0.573$, $p = 0.453$, $\eta^2 = 0.013$; July: $F_{1,37} = 0.010$, $p = 0.920$, $\eta^2 < 0.001$) (Fig. 3). The increase in the strength of the encapsulation response between October and February, shown in Fig. 2, is therefore most probably explained by seasonality but not by long-lasting immune priming. The homogeneity of slopes assumptions were fulfilled ($p \geq 0.750$).

Table 3. Long-lasting immune priming. Results of multivariate repeated measures ANCOVA for determining the change in the strength of the encapsulation responses between the primary and secondary immune challenges (sampling occasion), measured at different times of the year (season). Partial eta-squared (η^2) is reported as an estimate of effect size.

Within-subject effects	Wilks' λ	*F*	df	*p*	η^2
Source of variation					
Sampling occasion	1.000	0.014	1,80	0.908	< 0.001
Sampling occasion × season	0.838	5.146	3,80	0.003	0.162
Sampling occasion × log(body mass)	1.000	0.005	1,80	0.945	< 0.001
Between-subject effects		*F*	df	*p*	η^2
Source of variation					
Intercept		126.577	1,80	< 0.001	0.613
Season		1.066	3,80	0.368	0.038
Log(body mass)		3.199	1,80	0.077	0.038

Discussion

Endogenous seasonal variation in immune defence

The noble crayfish showed weaker encapsulation responses in October as compared with those in the other seasons, as well as a significant decrease in the strength of the encapsulation response between July and September in the following year despite the stable environment. These findings indicate that the strength of this cellular immune defence mechanism is under endogenous regulation in the noble crayfish, suggesting an endogenous seasonal (circannual) rhythm in immunity (Paul *et al*. 2008). This conclusion is further supported by the recurring weaker encapsulation response observed in the second autumn. To our knowledge, these results provide the first evidence on endogenous seasonal variation in the immune defence of invertebrates. Previously, similar endogenous seasonal variation and rhythms in immune defence have only been reported in vertebrates (Brock 1983, Kiank *et al*. 2007). For example, a stronger immune response to and enhanced survival from an immune challenge during winter as compared with those in summer was found in laboratory mice (Kiank *et al*. 2007).

In boreal environments, noble crayfish are exposed to prominent seasonal differences in environmental cues, including only few hours without daylight, warm water temperatures and high food availability during summer, and only a few hours of daylight, with cold temperatures and less food during winter. Hence, a proper timing of behavioural and physiological activities, especially those related to reproduction, is important. It has been suggested that nocturnal species that spend most of the light period in shelters and species that overwinter in burrows might not have sufficient access to important environmental cues, such as photoperiod, to time their behaviour and physiology accordingly, and consequently rely on biological clocks (Sharma 2003, Paul *et al*. 2008). In the wild, noble crayfish are mainly active during dusk and the dark period but spend the day in a shelter, e.g. in burrows (Lundberg 2004). Moreover, snow and ice cover, which remains for several months, keep the photoperiod and temperature constant relative to temperate environments without ice-cover. That could explain why noble crayfish probably possess endogenous timekeeping mechanisms.

One of the current main hypotheses explaining seasonal differences in immune defence predicts variation in immunity due to physiological trade-offs with other seasonal varying functions (Martin *et al*. 2008). More specifically, the allocation of energy to immune defence might decline in favour of other competing physiological processes during specific times, such as during the breeding or reproductive season (Martin *et al*. 2008, Schmid-Hempel 2011), and thus results in weakened immunity. Many previous studies in the wild or under natural conditions found support for the hypothesized resource allocating conflict between reproduction and immunity, reporting decreased immune defence during the reproductive period or during specific reproductive stages (e.g. fish: Kortet *et al*. 2003, bivalves: Duchemin *et al*. 2007, reptilians: French & Moore 2008). Therefore, it is possible that the endogenous seasonal variation we found in crayfish immune defence, i.e. the recurring endogenously driven decrease in the strength of the encapsulation response in autumn, is related to their reproduction, which in Finland occurs during September and October (Kilpinen 2003). Hence, a genetically programmed and self-sustained circannual clock might primarily regulate reproduction and indirectly influence the strength of immune defence.

Martin *et al*. (2008) previously pointed out that in vertebrates hormonal changes during the year, e.g. during reproduction, moulting or overwintering, probably mediate seasonal variation in immune defence. The same is possibly true for invertebrates (Rolff & Siva-Jothy 2003). Demas *et al*. (2011) recently highlighted that the nervous, endocrine and immune systems are not independent of each other but that those systems are highly connected in both vertebrates and invertebrates. Therefore, it might also be possible that the circannual clock directly regulates the nervous, endocrine and immune systems, and that our experimental crayfish underwent endogenous changes in both hormones and immune defence during autumn, independent of the energetic trade-off with reproduction.

As many other studies in invertebrates (Schmid-Hempel 2011), we also found evidence that, as compared with males, noble crayfish females had on average a stronger encapsulation response. Although several hypotheses have been proposed (*see* Schmid-Hempel 2011), the most probable explanation for the difference in the strength of immune defence between males and females may be related to sexually dimorphic life-history strategies (Rolff 2002). Trivers (1972) highlighted that the fitness of females, generally investing more in offspring, is limited by the ability to produce eggs, whereas the fitness of males, whose reproductive success usually varies more, is limited by the number of matings. According to Bateman's principle describing this discrepancy, males gain fitness by being competitive and increasing their mating success, and females, on the other hand, by longevity and increasing the number of reproductive seasons (Bateman 1948, Schmid-Hempel 2011). Therefore, females are predicted to invest more in their immune defence in order to increase their survival and consequently the number of reproductive seasons (Rolff 2002).

Although our results on endogenous seasonal variation in immune defence are the first reported in invertebrates, they are a logical continuation of earlier evidence, demonstrating that the immune defence of invertebrates follows other endogenous rhythms and is influenced by biological clocks of shorter time scales, such as circadian (e.g. in *Drosophila*: Lee & Ederyl 2008, Keller *et al*. 2009, Stone *et al*. 2012, or in the signal crayfish, *Pacifastacus leniusculus*: Watthanasurorot *et al*. 2011, Noonin *et al*. 2013) or even circatidal clocks (e.g. in the common shore crab, *Carcinus maenas*: Hauton *et al*. 1995). For example, Lee and Ederly (2008) reported that a circadian clock controls the strength of the immune response to and survival from a pathogenic infection in *Drospohila melanogaster*. They observed an identical pattern in survival when individuals were held under a 12:12 h light-dark cycle or in constant darkness, and, more specifically, found a stronger immune response and increased survival in case of nighttime infections (Lee & Ederyl 2008). In another freshwater crayfish species, two important immune defence parameters (the expression of prophenoloxidase and the number of circulating haemocytes) as well as survival from a pathogenic infection are also under circadian regulation (Watthanasurorot *et al*. 2011, Noonin *et al*. 2013).

Our study provides the first important step in the exploration of endogenous seasonal variation in one immune defence parameter of invertebrates. However, further studies that examine different immune defence parameters over several years in both immature and mature individuals are needed to provide stronger support for endogenous seasonal (circannual) rhythms in the immune defence of invertebrates, since a circannual rhythm is defined as a pattern that reoccurs in approximately 12 month cycles after isolation from environmental cues (Paul *et al*. 2008).

Long-lasting immune priming

There is evidence for long-lasting immune priming in invertebrate species (Moret & Siva-Jothy 2003, Schmid-Hempel 2005, Sadd & Schmid-Hempel 2006, Roth & Kurtz 2009, Rodrigues *et al*. 2010). Hence, we predicted to find a stronger encapsulation response to the secondary immune challenge with a nylon monofilament implant in the subsequent season as compared with that to the primary challenge, when the noble crayfish were held under stable laboratory conditions, and assuming that the implant causes a significant immunological challenge. However, we found no evidence for a previous challenge with a nylon monofilament implant enhancing the encapsulation response of the noble crayfish in subsequent seasons. This is because, when controlling for the effect of endogenous seasonal differences in immunity, the strength of the encapsulation response to the immune insult did not differ between naïve individuals and individuals that were pre-challenged two to four months before the secondary challenge occurred, independent of season.

These results suggest that in crayfish long-lasting immune priming is not present or at least not initiated by the immune insults we used. It is possible that the nylon insult only lead to a temporary activation of the immune system (Hauton & Smith 2007), often called non-specific immune

priming (Schmid-Hempel 2005). Alternatively, it might also be possible that the single challenge with a relatively small-sized nylon implant was not strong enough to induce the immune priming mechanisms. Therefore, further studies using other immune challenges and investigating various immune responses are necessary to draw final conclusions whether an immune challenge in a previous season could enhance the immune defence of the noble crayfish. Nevertheless, our results highlight the danger of misinterpreting results when the endogenous regulation of immunity is ignored. Without the naïve controls, one might have been easily tempted to call the increase in the strength of encapsulation between the repeated immune challenges in October and February real immune priming. Therefore, we strongly suggest considering both the effect of the repeated exposure to pathogens as well as the time effect, even when individuals are held under constant conditions, when investigating immune priming.

Acknowledgements

We thank the staff of the Experimental Unit of the University of Oulu for the maintenance of the crayfish and for help in the practical execution of the experimental procedures. We are grateful to Aki Puhka and Jani Koskimäki for their help in the experiments, as well as Simon Uphill for proofreading the manuscript and correcting the English. We would also like to thank two anonymous referees for their helpful comments. This research was supported by the Academy of Finland (project #127398), the Emil Aaltonen foundation, the Doctoral Programme in Wildlife Biology Research (LUOVA) and by the strategic funding of the University of Eastern Finland (Innovative Research Initiative project).

References

Ardia, D. R., Gantz, J. E., Schneider, B. C. & Strebel, S. 2012: Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. — *Functional Ecology* 26: 732–739.

Aydin, H., Kokko, H., Makkonen, J., Kortet, R., Kukkonen, H, & Jussila, J. 2014: The signal crayfish is vulnerable to both the As and PsI-isolates of the crayfish plague. — *Knowledge and Management of Aquatic Ecosystems* 413, 03, doi:10.1051/kmae/2014004.

Bateman, A. J. 1948: Intra-sexual selection in *Drosophila*. — *Heredity* 2: 349–368.

Best, S. M. & Kerr P. J. 2000: Coevolution of host and virus: The pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits. — *Virology* 267: 36–48.

Brock, M. A. 1983: Seasonal rhythmicity in lymphocyte blastogenic responses of mice persists in a constant environment. — *The Journal of Immunology* 130: 2586–2588.

Cerenius, L., Bangyeekhun, E., Keyser, P., Söderhäll, I. & Söderhäll, K. 2003: Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. — *Cellular Microbiology* 5: 353–357.

Cheng, W. & Chen, J. 2001: Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*. — *Fish & Shellfish Immunology* 11: 53–63.

Cheng, W., Juang, F., Li, J., Lin, M., Liu, C. & Chen, J. 2003: The immune response of the giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to *Lactococcus garvieae* in relation to the moult stage. — *Aquaculture* 218: 33–45.

Demas, G. E., Adamo, S. A. & French, S. S. 2011: Neuroendocrine-immune crosstalk in vertebrates and invertebrates: Implications for host defence. — *Functional Ecology* 25: 29–39.

Dissanayake, A., Galloway, T. S. & Jones, M. B. 2011: Seasonal differences in the physiology of *Carcinus maenas* (Crustacea: Decapoda) from estuaries with varying levels of anthropogenic contamination. — *Estuarine, Coastal and Shelf Science* 93: 320–327.

Dubovskiy, I. M., Whitten, M. M. A., Yaroslavtseva, O. N., Greig, C., Kryukov, V. Y., Grizanova, E. V., Mukherjee, K., Vilcinskas, A., Glupov, V. V. & Butt, T. M. 2013: Can insects develop resistance to insect pathogenic fungi? — *PLoS One* 8: e60248, doi:10.1371/journal.pone.0060248.

Duchemin, M.B., Fournier, M. & Auffret, M. 2007: Seasonal variations of immune parameters in diploid and triploid Pacific oysters, *Crassostrea gigas* (Thunberg). — *Aquaculture* 264: 73–81.

Engqvist, L. 2005: The mistreatment of covariate interaction terms in linear model analyses of behavioural and evolutionary ecology studies. — *Animal Behaviour* 70: 967–971.

Fox, J. 2002: *An R and S-plus companion to applied regression*. — Sage Publications, Thousand Oaks, California.

French, S. S. & Moore, M. C. 2008: Immune function varies with reproductive stage and context in female and male tree lizards, *Urosaurus ornatus*. — *General and Comparative Endocrinology* 155: 148–156.

Gruber, C., Kortet, R., Vainikka, A., Hyvärinen, P., Rantala, M. J., Pikkarainen, A., Jussila, J., Makkonen, J., Kokko, H. & Hirvonen, H. 2014: Variation in resistance to the invasive crayfish plague and immune defence in the native noble crayfish. — *Annales Zoologici Fennici* 51: 371–389.

Hauton, C. & Smith, V. J. 2007: Adaptive immunity in invertebrates: A straw house without a mechanistic foundation. — *Bioessays* 29: 1138–1146.

Hauton, C., Hawkins, L. E. & Williams, J. A. 1995: Cir-

catidal rhythmicity in the activity of the phenoloxidase enzyme in the common shore crab, *Carcinus maenas*. — *Comparative Biochemistry and Physiology B* 111: 347–352.

Hawley, D. M. & Altizer, S. M. 2011: Disease ecology meets ecological immunology: Understanding the links between organismal immunity and infection dynamics in natural populations. — *Functional Ecology* 25: 48–60.

Jussila, J., Makkonen, J., Vainikka, A., Kortet, R. & Kokko, H. 2014: Crayfish plague dilemma: how to be a courteous killer? — *Boreal Environment Research* 19: 235–244.

Keller, M., Mazuch, J., Abraham, U., Eom, G. D., Herzog, E. D., Volk, H., Kramer, A. & Maier, B. 2009: A circadian clock in macrophages controls inflammatory immune responses. — *Proceedings of the National Academy of Sciences of the United States of America* 106: doi: 10.1073/pnas.0906361106.

Kiank, C., Koerner, P., Kessler, W., Traeger, T., Maier, S., Heidecke, C. & Schuett, C. 2007: Seasonal variations in inflammatory responses to sepsis and stress in mice. — *Critical Care Medicine* 35: 2352–2358.

Kilpinen, K. 2003. *Suomen rapu: Ravun nousu, tuho ja tulevaisuus*. — Edita, Helsinki.

Kortet, R. & Vainikka, A. 2008: Seasonality of innate immunity; evolutionary aspects and latest updates. — In: Durand, M. & Morel, C. V. (ed.), *New research on innate immunity*: 13–45. Nova Science Publishers, Inc., NY.

Kortet, R., Taskinen, J., Sinisalo, T. & Jokinen, I. 2003: Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus* L. — *Biological Journal of the Linnean Society* 78: 117–127.

Krams, I., Daukste, J., Kivleniece, I., Krama, T. & Rantala, M. J. 2013: Previous encapsulation response enhances within individual protection against fungal parasite in the mealworm beetle *Tenebrio molitor*. — *Insect Science* 20: 771–777.

Kurtz, J. & Franz, K. 2003: Innate defence: evidence for memory in invertebrate immunity. — *Nature* 425: 37–38.

Lazzaro, B. P., Sceurman, B. K. & Clark, A. G. 2004: Genetic basis of natural variation in *D. melanogaster* antibacterial immunity. — *Science* 303: 1873–1876.

Le Moullac, G. & Haffner, P. 2000: Environmental factors affecting immune responses in Crustacea. — *Aquaculture* 191: 121–131.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P. & Aquacop 1997: Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: Protection against vibriosis. — *Fish & Shellfish Immunology* 7: 227–234.

Lee, J. & Ederyl, I. 2008: Circadian regulation in the ability of *Drosophila* to combat pathogenic infections. — *Current Biology* 18: 195–199.

Lundberg, U. 2004: Behavioural elements of the noble crayfish, *Astacus astacus* (Linnaeus, 1758). — *Crustaceana* 77: 137–162.

Martin, L. B., Weil, Z. M. & Nelson, R. J. 2008: Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. — *Philosophical Transactions of the Royal Society B* 321–339.

McTaggart, S. J., Wilson, P. J. & Little, T. J. 2012: *Daphnia magna* shows reduced infection upon secondary exposure to a pathogen. — *Biology Letters* 8: 972–975.

Moret, Y. & Siva-Jothy, M. T. 2003: Adaptive innate immunity? Responsive-mode prophylaxis in the mealworm beetle, *Tenebrio molitor*. — *Proceedings of the Royal Society B* 270: 2475–2480.

Mydlarz, L. D., Jones, L. E. & Harvell, C. D. 2006: Innate immunity, environmental drivers, and disease ecology of marine and freshwater invertebrates. — *The Annual Review of Ecology, Evolution, and Systematics* 37: 251–288.

Nelson, R. J., Demas, G. E., Klein, S. L. & Kriegsfeld, L. K. 2002: *Seasonal patterns of stress, immune function, and disease*. — Cambridge University Press, Cambridge, UK.

Noonin, C., Watthanasurorot, A., Winberg, S. & Söderhäll, I. 2013: Circadian regulation of melanization and prokineticin homologues is conserved in the brain of freshwater crayfish and zebrafish. — *Developmental & Comparative Immunology* 40: 218–226.

Paul, M. J., Zucker, I. & Schwartz, W. J. 2008: Tracking the seasons: the internal calendars of vertebrates. — *Philosophical Transactions of the Royal Society B* 363: 341–361.

Pauwels, K., De Meester, L., Put, S., Decaestecker, E. & Stoks, R. 2010: Rapid evolution of phenoloxidase expression, a component of innate immune function, in a natural population of *Daphnia magna*. — *Limnology and Oceanography* 55: 1408–1413.

Pham, L. N., Dionne, M. S., Shirasu-Hiza, M. & Schneider, D. S. 2007: A specific primed immune response in *Drosophila* is dependent on phagocytes. — *PLoS Pathogens* 3: e26, doi:10.1371/journal.ppat.0030026.

Pope, E. C., Powell, A., Roberts, E. C., Shields, R. J., Wardle, R. & Rowley, A. F. 2011: Enhanced cellular immunity in shrimp (*Litopenaeus vannamei*) after 'vaccination'. — *PLoS One* 6: e20960, doi:10.1371/journal.pone.0020960.

Rantala, M. J. & Kortet, R. 2004: Male dominance and immunocompetence in a field cricket. — *Behavioral Ecology* 15: 187–191.

Rantala, M. J. & Roff, D. A. 2007: Inbreeding and extreme outbreeding cause sex differences in immune defence and life history traits in *Epirrita autumnata*. — *Heredity* 98: 329–336.

Rodrigues, J., Brayner, F. A., Alves, L. C., Dixit, R. & Barillas-Mury, C. 2010: Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. — *Science* 329: 1353–1355.

Rolff, J. 2002: Bateman's principle and immunity. — *Proceedings of the Royal Society B* 269: 867–872.

Rolff, J. & Siva-Jothy, M.T. 2003: Invertebrate ecological immunology. — *Science* 301: 472–475.

Roth, O. & Kurtz, J. 2009: Phagocytosis mediates specificity in the immune defence of an invertebrate, the woodlouse *Porcellio scaber* (Crustacea: Isopoda). — *Developmental & Comparative Immunology* 33: 1151–1155.

Sadd, B. M. & Schmid-Hempel, P. 2006: Insect immunity shows specificity in protection upon secondary pathogen exposure. — *Current Biology* 16: 1206–1210.

Sadd, B. M. & Schmid-Hempel, P. 2007: Facultative but persistent transgenerational immunity via the mother's eggs in bumblebees. — *Current Biology* 17: R1046–R1047.

Schmid-Hempel, P. 2005: Evolutionary ecology of insect immune defences. — *Annual Review of Entomology* 50: 529–551.

Schmid-Hempel, P. 2011: *Evolutionary parasitiology: The integrated study of infections, immunology, ecology and genetics*. — Oxford University Press Inc., New York.

Sharma, V. K. 2003: Adaptive significance of circadian clocks. — *Chronobiology International* 20: 901–919.

Skurdal, J., Taugbøl, T., Burba, A., Edsman, L., Söderbäck, B., Styrishave, B., Tuusti, J. & Westman, K. 1999: Crayfish introductions in the Nordic and Baltic countries. — In: Gherardi, F. & Holdich, D. M. (eds.), *Crayfish in Europe as alien species: how to make the best of a bad situation*: 193–219. A. A. Balkema, Rotterdam.

Smilanich, A. M., Dyer, L. A. & Gentry, G. L. 2009: The insect immune response and other putative defenses as effective predictors of parasitism. — *Ecology* 90:1434–1440.

Smilanich, A. M., Mason, P. A., Sprung, L., Chase, T. R. & Singer, M. S. 2011: Complex effects of parasitoids on pharmacophagy and diet choice of a polyphagous caterpillar. — *Oecologia* 165: 995–1005.

Söderhäll, K., Cerenius, L. & Johansson, M. W. 1996: The prophenoloxidase activating system in invertebrates. — In: Söderhäll, K., Iwanaga, S. & Vasta, G. R. (eds.), *New Directions in Invertebrate Immunology*: 229–253. SOS Publications, Fair Haven.

Souty-Grosset, C., Holdich, D. M., Noël, P. Y., Reynolds, J. & Haffner, P. 2006: *Atlas of crayfish in Europe*. — Muséum National d'Histoire Naturelle, Paris.

Stone, E. F., Fulton, B. O., Ayres, J. S., Pham, L. N., Ziauddin, J. & Shirasu-Hiza, M. M. 2012: The circadian clock protein timeless regulates phagocytosis of bacteria in *Drosophila*. — *PLoS Pathogens* 8: e1002445, doi: 10.1371/journal.ppat.1002445.

Tidbury, H. J., Pedersen, A. B. & Boots, M. 2011: Within and transgenerational immune priming in an insect to a DNA virus. — *Proceedings of the Royal Society B* 278: 871–876.

Trivers, R.L. 1972: Mother-offspring conflict. — *American Zoologist* 12: 648–648.

Trowsdale, J. & Parham, P. 2004: Mini-review: defense strategies and immunity-related genes. — *European Journal of Immunology* 34: 7–17.

Unestam, T. 1972: On the host range and origin of the crayfish plague fungus. — *Report of the Institute of Freshwater Research, Drottningholm* 52: 192–198.

Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A. & Zenteno, E. 2009: Immunity mechanisms in crustaceans. — *Innate Immunity* 15: 179–188.

Watthanasurorot, A., Söderhäll, K., Jiravanichpaisal, P. & Söderhäll, I. 2011: An ancient cytokine, astakine, mediates circadian regulation of invertebrate hematopoiesis. — *Cellular and Molecular Life Sciences* 68: 315–323.

Westman, K., Savolainen, R. & Julkunen, M. 2002: Replacement of the native crayfish *Astacus* astacus by the introduced species *Pacifastacus leniusculus* in a small, enclosed Finnish lake: a 30-year study. — *Ecography* 25: 53–73.

Wikelski, M., Martin, L. B., Scheuerlein, A., Robinsoni, M. T., Robinson, N. D., Heim, B., Hau, M. & Gwinner, E. 2008: Avian circannual clocks: Adaptive significance and possible involvement of energy turnover in their proximate control. — *Philosophical Transactions of the Royal Society B* 363: 411–423.