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Short communication

Multigenerational immune priming in an invertebrate parthenogenetic *Artemia* to a pathogenic *Vibrio campbellii*

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1. Introduction

Invertebrates were traditionally thought to rely on the innate arm of the defense system to fight against pathogens [1,2], and adaptivity or memory of immune responses were previously considered the hallmark of a highly evolved immune system, only present in vertebrates [3]. However, there are now evidences indicating that invertebrates have immunological specificity and memory of a kind that is a functional equivalent of adaptive immunity in vertebrates [4]. These claims for adaptive-like immunity in invertebrates were based largely on a few (phenomenological) observations that previous exposure to pathogen and/or immune elicitor (like lipopolysaccharide, heat shock protein, glucan) has a positive impact on the immune system components [5–8] and on the resistance of the animals against subsequent microbial attacks [9,10]. This form of memory in an invertebrate is termed 'immune priming', which is broadly defined as increased protection to a pathogen following previous exposure to a pathogen or an immune elicitor. A few recent studies have indicated that similar to vertebrate adaptive immunity, the immune priming phenomenon in invertebrates is transgenerational i.e., the protective/immune responses are passed from parents to offspring [5,8,11]. For example, in the insect *Trichoplusia ni*, offspring from mothers that had been raised on a bacteria-rich diet had an increased immune response in terms of immune enzyme activity, and the expression of immune-related genes [12]. In addition, in the red flour beetle, *Tribolium castaneum*, it was shown that after parental exposure to heat-killed bacteria, transgenerational immune priming occurs through fathers as well as mothers [10]. However, an alternate explanation for the above-mentioned evidences/findings could be selection rather than paternal/maternal transfer of immune-priming traits [13]. In addition, in most of the previous studies on transgenerational immune priming in invertebrates, assessments of the maternally/paternally-transferred acquired immune responses or disease-resistant traits were carried out only in the first-generation progeny. If there is immune memory in invertebrates, it can be expected that the acquired memory-like immune responses or disease-resistant traits in the parental generation are transmitted across subsequent generations. However, at present, little evidence appears in the literature that unequivocally demonstrates the transfer/persistence of (immune) priming effects across subsequent generations.

Here, we used the well-developed host-pathogen laboratory model system, apomictic parthenogenetic *Artemia* (an aquatic invertebrate) and its pathogenic bacteria *Vibrio campbellii* strain LMG21363 to address the phenomenon of multigenerational immune priming in invertebrate. The pathogen *V. campbellii* was selected because it is an opportunistic bacterium that causes significant mortalities in the farmed aquatic animals, including *Artemia* [2]. In particular, we immune challenged a population of apomictic parthenogenetic *Artemia* (obtained from a single female) by exposing the population at early life stages to *V. campbellii* and examined the resistance of the three successive generation progenies (none of them were immune challenged) by challenging with the same strain of bacteria. We demonstrated for the first time in a crustacean/invertebrate that immune-challenged *Artemia* by specific bacteria bestow their next generation progenies with increased resistance against the same strain of bacteria and this acquired strain-specific immunity is further transmissible to successive generation progenies.

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2. Materials and methods

A population of apomictic parthenogenetic *Artemia* was obtained ovoviviparously from a single tetraploid female of Megalon Embolon saltworks in Greece [14]. The animals were grown till adult in 35 g l^{-1} artificial seawater under controlled laboratory conditions as described previously [15]. Throughout the culture period, the animals were fed *ad libitum* everyday with live green microalgae *Tetraselmis suecica* obtained from the Culture Collection of Algae and Protozoa (CCAP) Department (Dunstaffnage Marine Laboratory, Oban, UK). Under non-optimal environmental conditions, such as high salinity or low oxygen, parthenogenetic *Artemia* switch from an ovoviviparous to an oviparous mode of reproduction. To induce the *Artemia* to produce cysts, the salinity of the culture water was gradually increased from 35 to 80 g l^{-1} over a period of 10 days. Deposited cysts enter in a state of diapause and are not ready for hatching [16]. To terminate cyst diapause, the collected cysts were dehydrated in a saturated NaCl brine solution and exposed to a temperature of −20 °C for 3 months, and then stored as activated cysts at 4 °C until use. For the experiment, the cysts were hatched under axenic conditions (to avoid or minimize the initial load of bacteria in the culture condition) via decapsulation using NaOH (32%) and NaOCl (50%) as described previously [17]. The sterile decapsulated cysts were transferred to 1-l glass bottles containing 35 g l^{-1} of sterile artificial seawater. Following incubation at 28 °C under constant illumination for 48 h (or 2 days posthatching, dph), the emerged instar II larvae (stage at which mouth is open for ingestion of food) were used for experimental treatments.

A group of 800 instar II nauplii (2 dph) was distributed in two groups (treatment and control), each with 3 replicates. Each group was maintained in a 1-l glass bottle containing sterile artificial seawater (35 g l^{-1}), maintained at 28 °C under constant illumination (approximately 27 μmol m^{-2} s^{-1}) and aeration. The treatment groups (on 6 dph) were exposed to 10^7 cells ml^{-1} concentration of *V. campbellii* strain LMG21363 for a period of 3 days. On 10 dph, the nauplii from the bottles were collected over a sieve (250 μm), rinsed several times with sterile seawater to wash away the bacteria associated with the nauplii, re-suspended in a new sterile 1-l glass bottles that contained sterile artificial sea water, and then again exposed to the same concentration of *V. campbellii* for another 3 days. On 14, 15 and 16 dph, the nauplii were washed in a similar way as described above to remove the bacteria. The control group, unexposed to *V. campbellii*, went through the same handling process. The *V. campbellii* exposure to *Artemia* nauplii was continued for 13 days ahead of the reproductive period (under standard laboratory conditions, *Artemia's* uterus develop on day 16 posthatching). Only early life stages of *Artemia* were exposed to *V. campbellii* to ensure that the uterus carrying the cysts/embryos was not directly exposed to the pathogenic stress conditions. On 17 dph, the salinity of the rearing water was increased from 35 g l^{-1} to 60 g l^{-1} in order to inhibit the growth of *V. campbellii* and after 2 days, the salinity was further increased to 80 g l^{-1} to instigate cysts production.

Approximately 28 days posthatching, adult females (F0 generation) in both control and treatment started producing cysts (F1 generation). The F1 cysts from 3 replicates were isolated, pooled, and after terminating diapause by proper conditioning, were axenically hatched as described above. A major part of the emerged F1 nauplii were further cultured to maturity, after which the F2 nauplii were collected. The experiment was continued until, and including, the F3 generation. During the culture period, live algae were fed *ad libitum* every day, and to minimize bacterial exposure/contamination, sterile glass bottles and sterile artificial seawater were used. The detailed experimental design is shown schematically in Fig. 1. An aliquot of the emerged (F1 to F3) nauplii at instar 11 stage collected during each generation were subjected to *V. campbellii* challenge tests as described previously [15]. In brief, groups of 20 larvae were transferred in 7 replicates into separate sterile 40-ml glass tubes that contained 30 ml of 35 g l^{-1} sterile seawater. The nauplii were challenged with *V. campbellii* at 10^7 cells ml^{-1}. The survival of *Artemia* was scored at every indicated time intervals by counting the live *Artemia* as previously described [15].

At each generation, survival data were analyzed for statistical differences by subjecting the data to logistic regression analysis using GenStat (VSN international) version 16. Significance level was set at $P < 0.05$.

3. Results and discussion

In this study, we tested the phenomenon of multigenerational immune priming in an invertebrate, with the aim to seek evidence of adaptive-like immunity in invertebrates. For that, we exposed a population produced by an apomictic parthenogenetic *Artemia* i.e., lineages derived from a single mother, to a specific bacterial strain and evaluated the resistance of the offspring for three successive generations towards the same bacterial strain. Our results showed that the T-F1 offspring of F0 *Artemia* that were exposed at early stages to *V. campbellii* exhibited a significantly higher survival when challenged to *V. campbellii* than did the respective progeny of control C-F1 *Artemia* (Fig. 2A). Interestingly, this relatively higher survival extended to *Vibrio*-challenged T-F2 and T-F3 progenies,

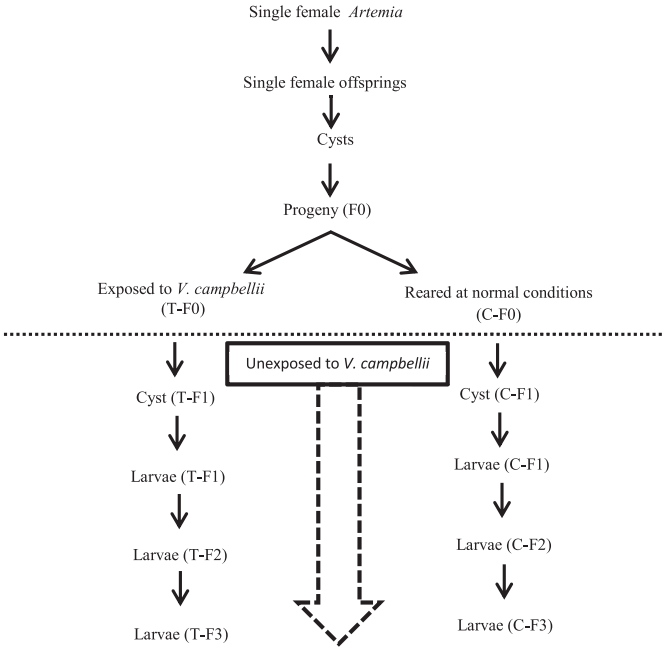

Fig. 1. Scheme of the experiment. A single female parthenogenetic *Artemia* was propagated to the next generation (i.e., single female offspring) under normal growth conditions. The single female offspring (all females) were reared normally until adulthood, and were then induced to produce cysts (F0). On hatching, the F0 progeny were divided into 2 groups. One group was exposed to *V. campbellii* ahead of the reproductive period (T-F0) as described in the methodology. The other group was grown unexposed, under normal culture conditions 28 °C (C-F0). Approximately 28 days posthatching, the parental (F0) females from the treatment (T-F0) and control (C-F0) groups produced their next generation cysts i.e., T-F1 and C-F1, respectively. The cysts were hatched to produce their corresponding larvae i.e., T-F1 and C-F1. The F1 larvae from both groups were further cultured isothermally at 28 °C, without being given *V. campbellii* exposure, to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation.