Gammarus: Important Taxon in Freshwater and Marine Changing Environments

Guest Editors: Almut Gerhardt, Michelle Bloor, and Chris Lloyd Mills
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Gammarus spp. consist of more than 100 freshwater, brackish, and marine species in the Northern hemisphere. They represent important keystone species in aquatic ecosystems and are often present in high abundance. As shredders and detritus feeders, they contribute to the detritus cycle and the microbial loop. Gammarids are also carnivorous, feeding on small invertebrates and carrion. Due to their widespread distribution, significance in the food web, and sensitivity to a wide range of pollutants, they are important bioindicators for water quality assessment.

Gammarus spp. are ecologically highly successful due to the following characteristics: wide trophic repertoire and foraging plasticity, migration ability and tendency to drift, which allows them to easily invade and colonize ecosystems, high reproductive capacity with several broods per female per year, and a high number of offspring and relatively longevity (1-2 yrs).

Gammarus spp. and their American relative Hyalella azteca are standard test species in ecotoxicity testing in the USA and UK. A new OECD test guideline is currently being prepared for gammarids, which will consist of a variety of in situ and ex situ ecotoxicological studies based on different measurement parameters.

The evolution of gammarids is particularly interesting: gammarids contain several Ponto-Caspian and Atlantic invasive species, which have spread throughout Europe. Some species are currently being divided into several geographical forms. Although the genome of Gammarus pulex has yet to be sequenced, 12345 expressed sequence tags are known, which might be the basis for future innovative knowledge in taxonomy and toxicogenomics.

Despite the taxon’s importance in different fields of biology (e.g., ecology, evolution, ecotoxicology, taxonomy, biogeography), its application (as test species and bioindicators) in scientific research tends to consider isolated questions. The aim of this special issue on Gammarus spp. is to highlight the importance of this taxon and facilitate interdisciplinary research. The focus of this issue is to present articles that showcase different facets of gammarid research, including methods that demonstrate direct practical approaches. In order to reach a wide spectrum of readers from different disciplines and geographical regions, we chose this journal with global open access.

Gammarids are known to be important shredders in the aquatic detritus cycle and the microbial loop. The most recent research within the field of gammarid-microbial interactions is summarized and discussed in the first article by D. Nelson. Gammarids prefer so-called conditioned leaves as food, that is, leaves colonized by a microbial biofilm. The role of hyphomycete fungi as a source of secondary metabolites is stressed in the paper. However, although the effects of leaf type and microbial colonization on Gammarus spp. feeding activity, growth, and survival has been studied, the effects of Gammarus spp. on shaping the microbial community still remain unknown.

Understanding dietary requirements of gammarids is essential knowledge to describe feeding schemes for a
successful laboratory culture. M. Bloor tests unconditioned and conditioned (either naturally conditioned or artificially conditioned) alder leaves as a food source for *Gammarus pulex* and *Asellus aquaticus* and concludes that both species prefer naturally conditioned leaves compared to artificial conditioning. M. Bloor provides a simple method to prepare alder leaves for laboratory culturing of both species. Successful breeding is an essential requisite for a standard test species in ecotoxicological test procedures.

A. Gerhardt presents a simple low-cost test system with *Gammarus* spp. studying survival, feeding, and behaviour as well as biomarkers, to be used both *in* and *ex situ*. This test protocol does not need sophisticated laboratory equipment and hence can be applied worldwide. Compared to the standard test species *Daphnia magna*, gammarids tend to be as sensitive towards toxicants and are more ecologically relevant test species. We hope to increase the application of gammarids in aquatic ecotoxicology by providing simple methods for breeding, feeding, and test systems.

Whilst gammarids can be very sensitive towards pesticides, they are also susceptible to natural defence substances released by aquatic plants. M. J. Dixon and P. J. Shaw show that both pulses of wash water from a watercress farm and isolated phenethyl isothiocyanate (PEITC) can influence the reproductive behaviour of *Gammarus pulex*. Short-term pulses lead to the interruption of precopula pairs, with recovery occurring in clean water. Such a transient behavioural parameter has the potential to be used as an early warning indicator of pulsed pollution stress.

A sound basis for every ecological and ecotoxicological study is species determination. In gammarids several cryptic species have been found based on genetic methods. However, the proof of mechanisms in speciation such as geographical and/or ecological isolation is still lacking. Do genetic differences suffice to describe (sub)species, or do we need a combination of genetic, morphological, and ecological methods? Haug et al. present the most recent methods for imaging gammarids from whole animals to tiny structures using both high- and low-cost techniques. Modern imaging methods in combination with sophisticated software are very powerful tools which facilitate taxonomic determination. In this paper, different techniques are presented, from simple light microscopy with staining techniques, autofluorescence, and polarized light applications to focused ion beam scanning electron microscopy, confocal laser microscopy, and microtomography.

Although most gammarids represent freshwater *Gammarus* species, brackish and marine gammarids have recently been studied in more detail. L. Delgado et al. show that both adult and juvenile *Gammarus aequicauda* have a wide salinity tolerance range and that juvenile growth rate is only affected towards the extremes of the salinity tolerance range. Ecological plasticity is a prerequisite to spread to different habitats and geographical locations. Gammarids are highly successful in this aspect.

A. Mirzajani et al. describe selection of gammarid species for potential use as a natural food source for warm water fish farming in Iran. In this interesting approach the reproductive traits of 7 amphipod species have been studied with respect to their breeding periods and reproductive output. They identified 4 species that might be suitable for warm water fish aquaculture.

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Review Article

Gammarus-Microbial Interactions: A Review

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Received 14 March 2011; Revised 4 May 2011; Accepted 19 May 2011

Academic Editor: Almut Gerhardt

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Gammarus spp. are typically classified as shredders under the functional feeding group classification. In the wild and in the laboratory, Gammarus spp. will often shred leaves, breaking them down into finer organic matter fractions. However, leaf litter is a poor quality food source (i.e., high C:N and C:P ratios) and very little leaf material is assimilated by shredders. In freshwater habitats leaf litter is colonized rapidly (within ~1-2 weeks) by aquatic fungi and bacteria, making the leaves more palatable and nutritious to consumers. Several studies have shown that Gammarus spp. show preference for conditioned leaves over nonconditioned leaves and certain fungal species to others. Furthermore, Gammarus spp. show increased survival and growth rates when fed conditioned leaves compared to non-conditioned leaves. Thus, Gammarus spp. appear to rely on the microbial biofilm associated with leaf detritus as a source of carbon and/or essential nutrients. Also, Gammarus spp. can have both positive and negative effects on the microbial communities on which they fed, making them an important component of the microbial loop in aquatic ecosystems.

1. Introduction

The diets of amphipods in the genus Gammarus are variable [1]. For example, Gammarus spp. can serve as detritivores [2, 3], herbivores [4, 5], predators [6, 7], and even cannibals [2, 8, 9] in aquatic ecosystems. However, under the functional feeding group classification [10–12], Gammarus spp. are typically classified as shredders/facultative shredder collectors [1]. In the wild and in the laboratory, Gammarus spp. often function as shredders consuming leaves and other coarse particulate organic matter (CPOM), breaking it down into smaller fractions or fine particulate organic matter (FPOM). Microbes, such as bacteria and fungi, are often associated with particulate organic matter such as leaves and decaying wood [13, 14]. Leaf detritus, in particular, is an important carbon source for the microbial loop in aquatic ecosystems [13]. Leaf matter serves as a substrate for bacterial and fungal growth, while at the same time supplying the microbial community with carbon in the form of leached dissolved organic carbon (DOC) [13]. Along with physical abrasion and soluble organic matter leaching, microbial decomposition and invertebrate feeding interact to regulate leaf litter breakdown rate in aquatic ecosystems [15]. Detritus-associated bacteria and fungi are responsible for detrital decomposition and its increase in palatability and nutritional quality to consumers [11, 16, 17]. Invertebrate consumers often rely on the microbial biofilm as a carbon source rather than on the detritus itself [11, 14, 18]. Cummins [2] refers to CPOM as a “cracker” and its associated microbes as “peanut butter.” The CPOM (cracker) acts as a vessel, enabling the consumer to more easily ingest the more nutritious bacteria and fungi (peanut butter).

Over the years, research has shown that Gammarus spp. feed on conditioned or inoculated detritus (i.e., leaves, leaf discs, or sediment) with “suitable” microflora [17, 19–22]. In addition, research has shown higher survival and growth rates of Gammarus amphipods in the laboratory when they are fed leaves with fungal growth compared to unconditioned or sterile leaves [20, 23, 24]. Freshly shed and sterile leaves typically have low nutritive value (i.e., high C:N and C:P ratios) and contain high amounts of lignin and cellulose, which are virtually indigestible to most invertebrates [25]. Therefore, for shredders, the percentage of food ingested and converted into invertebrate biomass is typically very low. As a result, many shredders, including Gammarus amphipods, wait until microbes (which are
typically highly nutritious) colonize and build up on this poorly nutritious food before feeding.

Gammarus spp. have also been shown to have both negative and positive effects on the microbial communities on which they feed, illustrating the importance of this genus to the microbial loop in lotic and lentic ecosystems. Most research investigating interactions between microbes and invertebrates has been focused on the role of microbes as a potential food source [26]. Although relatively little is known of the feedback effects that grazing invertebrates, such as Gammarus amphipods, can have on their microbial food [26], it has been demonstrated that microbial metabolism, production, and biomass can be influenced by both “bottom-up” and “top-down” controls [27–29]. Although invertebrate feeding can decrease microbial biofilm biomass, it has also been shown to stimulate microbial growth and activity [27, 30]. Thus, Gammarus spp. are often involved in a feedback loop with the microbial community on which they feed. In some cases, these feedbacks can be positive [28–30], while in others, they can be negative [29].

The specific objective of this review is to evaluate what is known regarding how microbes influence Gammarus spp. feeding preference, survival, and growth in the laboratory and aquatic habitats. In addition, it will be discussed how Gammarus spp. affect the microbial community on which they feed, either through ingestion or other types of interactions. Finally, the current state of research investigating Gammarus-microbial interactions will be reviewed and possible future research directions will be discussed.

2. Food Selection, Survival, and Growth

Quality of detritus is an important factor that determines food selection by shredders. Research has shown that shredders tend to prefer certain leaf species to others [31–33] and conditioned leaves over non-conditioned leaves [33–40]. Typically, shredders select food based on several characteristics of leaves, which include toughness, nutrient content, and the degree of conditioning by microbes [40]. Gammarus spp. are no exception [19, 20, 23, 31, 33, 41]. In some of the earliest laboratory experiments investigating food selection by Gammarus spp., Bärlocher and Kendrick [19] investigated food (leaf species) and fungi preference of Gammarus pseudolimnaeus. When very little microflora were present on leaf discs, G. pseudolimnaeus preferred ash to maple and maple to oak leaves. Bärlocher and Kendrick [19] then presented amphipods with pure colonies of ten different hyphomycetes along with maple leaf discs with very little associated microflora. Gammarus pseudolimnaeus always preferred the fungus to the leaf discs and in several cases the amphipods entirely ignored the leaves and consumed only the hyphomycetes.

As Bärlocher and Kendrick [19] demonstrated, G. pseudolimnaeus can exhibit preference for certain conditioned leaf species over others. Other Gammarus spp. have shown similar preferences. In the laboratory, the stygophilic G. troglphilus consumed conditioned oak if they were the only leaves presented to it [31]. However, if presented with conditioned oak and elm leaves simultaneously, G. troglphilus ingested the elm leaves and ignored the oak. Pöckl [23] simultaneously offered G. fossarum and G. roeseli eight different naturally decaying (i.e., conditioned) leaf species. The most preferred and quickly eaten were leaf discs of lime, ash, and alder. Both species showed little interest in oak leaves, and beech leaf discs were nearly untouched [23]. This behavior most likely resulted from differences in toughness of the leaves, leaf thickness, and chemical constituents (e.g., phenols and tannins) [23].

To determine if G. minus could distinguish different foods and exhibit a preference for the different foods, Kostalos and Seymour [20] performed a series of laboratory and field experiments. They individually compared preference of five different foods against a control, which contained a microflora most similar to fresh stream leaves [20]. The five different foods consisted of elm leaves with no microflora (sterile), bacteria-enriched elm leaves, conditioned elm leaves with a reduced bacterial fauna (still containing fungi), fungus-enriched (Tetrachaetum elegans) elm leaves, and the fungus T. elegans alone. Gammarus minus most strongly preferred the fungus-enriched leaves and conditioned leaves with a reduced bacterial fauna to the control leaves. The sterile elm leaves were least preferred.

In another laboratory study, Friberg and Jacobsen [41] examined the feeding preferences of G. pulex. Overall, G. pulex preferred conditioned alder leaves over five other food items which included conditioned beech leaves, fresh beech leaves, Sitka spruce needles, a fresh macrophyte, and a fresh filamentous green algae. The authors found no linear relationships between food preference and fiber content, toughness, phosphorous content, nitrogen content, and C:N ratio, leading them to believe that bacterial or fungal coating was responsible for the preference patterns. In another study using G. pulex, Graça et al. [42] demonstrated that when offered a choice between unconditioned leaf discs of elm, leaf discs of elm inoculated with the fungus Anguillospora longissima, or A. longissima mycelia, G. pulex was able to discriminate between the different foods and concentrated its feeding on the inoculated leaf discs, and to a lesser extent, on the unconditioned leaf discs. The A. longissima mycelia were ignored by G. pulex. Because food preference was not correlated with fungal biomass, leaf disc toughness, leaf decomposition, or nitrogen content, Graça et al. [42] concluded that other unmeasured factors could have affected food preference by G. pulex. These could include the fungal synthesis of micronutrients or the differential ability of fungi to eliminate plant allelochemicals among others [42].

Gammarus spp. have also been shown to prefer particular fungal species to others. When offered leaves colonized separately by one of eight species of aquatic hyphomycetes, Arsuffi and Suberkropp [17] found Gammarus amphipods to be highly selective feeders. Leaves colonized by the fungus Alatospora acuminata were the most preferred, but Gammarus also fed on leaves colonized by Clavariopsis aquatica and Flagellospora curvula. Feeding on other aquatic hyphomycetes was negligible [17]. Aquatic hyphomycetes produce secondary metabolites that function in microbe-microbe interactions and may also defend the fungi from invertebrate feeding. Arsuffi and Suberkropp [17] suggest
that secondary metabolites from fungi are responsible for the variation observed in feeding preferences, growth rates, and survivorship of shredders consuming leaves colonized by different fungi [17].

The combination of leaf and fungal species has also been shown to influence selection by Gammarus spp. In a laboratory study, individuals of G. tigrinus were given a choice between six different leaf/fungus combinations [21]. The leaf discs were conditioned with single species of aquatic hyphomycetes and their concentrations of proteins, lipids, and ergosterol (an indicator of fungal biomass) were measured. Although total consumption was not correlated to the lipid or protein content of the leaves or the fungal biomass, G. tigrinus showed a slight preference for some leaf/fungal combinations over others [21]. The authors then extracted fungal mycelia and applied the extracts to unconditioned leaf discs. Gammarus tigrinus preferred naturally conditioned leaf discs to the extract-coated leaf discs, suggesting that natural colonization over time makes the leaf/fungi combination more attractive compared to a rapid assembly of the parts.

In a more recent study, Assmann and Elert [22] examined the role of fungal attractants and repellents in food preference of the amphipod G. roeseli. Because both attractants and repellents seemed to act on G. roeseli feeding preference, the authors suggest that the relative ratios of repellents and attractants might determine consumption of fungi by Gammarus. Furthermore, changes in the environment could lead to changes in the relative ratio of attractants to repellents [22]. Thus, food preference may be governed by environmental conditions rather than being fixed in the consumer.

Amphipods fed conditioned leaves and/or fungi have increased assimilation efficiencies. Low assimilation efficiency results in less matter and energy available for maintenance, growth, and reproduction [43], thus compromising performance. Bärlocher and Kendrick [44] compared the assimilation efficiencies of G. pseudolimnaeus fed elm leaves, maple leaves, or the mycelium of one of ten fungi (5 aquatic hyphomycetes and 5 terrestrial hyphomycetes). Although the amount of food consumed was ten times greater in all of the leaf diets than in the fungi diets, the highest assimilation efficiencies were found for those individuals fed four of the ten fungi. Only 10% of the dry mass, 14–18% of the protein, and 17–19% of the energy of either elm or maple leaves were assimilated by the amphipods. However, G. pseudolimnaeus assimilated approximately 43–76% of the dry mass, 73–96% of the protein, and 70–83% of the energy when fed fungal mycelium commonly found in streams [44].

Research has shown higher survival and growth rates when Gammarus spp. are fed conditioned leaves compared to non-conditioned or sterile leaves. In addition to their experiments on food preference, Kostalos and Seymour [20] experimentally tested the survival of G. minus on ten different diets. These experiments showed significant differences in survivorship over a ten-week period, with the highest survivorship (45–88%) occurring on fungus-enriched leaves [20]. Intermediate survival rates (36–63%) occurred on leaves with a viable bacterial flora while the lowest survivorship (~3%) occurred on leaves that had no or a reduced microflora [20]. Other Gammarus spp. have shown higher growth rates when fed conditioned leaves. Graça et al. [33] found that conditioning had a significant effect on the growth of G. pulex. Similarly, Pöckl [23] found that neonates, juveniles, and early adults of G. fossarum and G. roeseli fed leached and decaying leaves of lime, elm, and hornbeam with surface growth of aquatic fungi and bacteria had higher growth rates than amphipods fed fresh, growing leaves. These studies suggest that microbes, particularly fungi, confer an advantage to Gammarus spp. by positively influencing survival, growth rates, or both.

In contrast, Graça et al. [24] found no significant increase in the survival of G. pulex on fungally conditioned leaf material when compared to unconditioned food. In general, survival of G. pulex was low on both conditioned and unconditioned leaves [24]. Although growth rates were higher on conditioned leaf material, the difference was not significant [24]. The authors offered an explanation for this lack of significance, using the results of an energy budget study. Individuals of G. pulex feeding on unconditioned leaves had a significantly lower respiration rate than those individuals feeding on conditioned leaves. The authors hypothesized that the lower metabolic demands as a result of a lower respiration rate compensated for the reduced energy intake. Thus, G. pulex is able to maintain a constant growth rate, even when food quality is poor.

3. Effects of Feeding on the Microbial Community

The effect Gammarus spp. have on microbial communities is not well known. Obviously, Gammarus amphipods can influence microbial biomass and production through mechanical removal (i.e., direct consumption). Direct consumption of biofilms by invertebrates has been shown to decrease microbial biomass and alter microbial community composition [45–49], however, consumption has also been shown to stimulate microbial growth [27, 30]. Shredding of leaves by Gammarus spp. may enhance microbial respiration by increasing the surface area of the leaf, which can lead to higher microbial respiration per unit mass of leaves [30]. In addition, increased fragmentation of leaves and excretion by Gammarus amphipods may lead to an increase in the availability of DOC and inorganic nutrients [30]. Thus, if a biofilm is nutrient limited, leaf shredding by Gammarus spp. can possibly relieve nutrient limitation constraints. Direct consumption by Gammarus spp. can not only directly decrease microbial biomass, but it can also change biofilm architecture, thus altering the delivery of inorganic nutrients and energy to the biofilm [29, 49]. Morrison and White [27] showed that microbial biomass was higher on detritus (conditioned oak leaves) that had been grazed by G. mucronatus than on ungrazed detritus. In addition to increasing microbial biomass, grazing by G. mucronatus increased metabolic activity and changed microbial community structure [27]. As amphipods grazed, microbial community structure shifted from one with both prokaryotes (bacteria) and microeukaryotes (fungi) to one dominated by bacteria [27]. Because microbial biofilms are
important mediators of energy flux and nutrient transformation in aquatic habitats, changes in microbial biomass, community composition, and biofilm architecture may have profound effects on aquatic ecosystem functioning [50, 51].

More recently, Kinsey et al. [30] compared the influence of feeding by cave and surface forms of G. minus on microbial biofilms and found that both forms increased the respiration rate of leaf-associated microbes by 32–52%. However, the cave form had a 15% greater stimulatory effect on microbial respiration. Kinsey et al. [30] concluded that their results may have been due to an attraction of G. minus to leaves with greater microbial growth or due to the amphipods stimulating microbial respiration by (1) increasing the availability of DOM and inorganic nutrients through fragmentation and excretion, (2) increasing water flow over the microbial biofilm, thus reducing boundary layer effects and increasing diffusion rates of nutrients and oxygen into biofilms, or (3) increasing leaf surface area, thereby increasing microbial respiration per unit mass of leaves. Cooney and Simon [29] then used microcosm experiments to examine how bacterial production on rocks and fine sediments from cave streams responded to amendments of dissolved organic matter (DOM) and to the cave form of G. minus. Interestingly, feeding by G. minus strongly suppressed bacterial production on rocks but had no effect on bacterial production on fine sediments. In addition, microbial production on rocks was stimulated by DOM amendments but production on sediments was not. Their results indicate that both resources and consumers play important roles in regulating microbial activity, particularly on rocky substrates.

4. Conclusions

This paper illustrates the importance of bacteria and fungi in the diet of Gammarus amphipods. It has been shown that Gammarus spp. frequently prefer certain leaf species to others and conditioned leaves to unconditioned leaves. Conditioning of detritus often enhances survival and even growth of Gammarus amphipods. Furthermore, Gammarus can have a significant influence on microbial communities through consumption of microbially enriched detritus, particularly fallen leaves. Although there is a body of literature on the interactions between Gammarus spp. and microbes, the full story is not complete. As important as Gammarus spp. are to detrital processing and nutrient cycling in aquatic ecosystems, there seems to be a decrease in interest in their interactions with microbes. Given the general paucity of recent information on Gammarus performance (e.g., survival, growth, and fecundity) after being fed bacteria and/or fungi, there should be a renewed interest in research on Gammarus-microbial interactions. More specifically, stoichiometric theory and unsaturated fatty acid analysis have been used by researchers to examine energy flow and growth efficiency in a number of aquatic consumers [52–55].

Future research should address stoichiometric relationships between Gammarus spp. and “conditioned” detritus. Colonization by microbes influences C:N and C:P ratios of leaf litter [56]. Furthermore, consumers often have lower C:N and C:P ratios than their food, thus elemental imbalances between detritivores (e.g., shredders) and their food can be common [53, 56–58]. An inadequate supply of one or more nutrients can constrain animal growth and alter their life history [57]. One way in which to examine the nutrient deficiency in consumers is the threshold elemental ratio (TER). Threshold elemental ratios are elemental ratios at which growth limitation of a consumer switches from one element to another [52, 57]. Calculation of TERs (C:N and C:P) requires estimates of assimilation efficiencies for C, N, and P, ingestion rates, respiration rates, and %C, %N, and %P of consumers. When the TER of the consumer is equal to the C:nutrient ratio of the consumer’s food, animal growth is limited by both C and the nutrient [53]. When the TER of the consumer deviates from the C:nutrient ratio of the food, either C or the nutrient is limiting [53]. Further elucidation of the importance of highly nutritious microbes in Gammarus diets could be provided by identifying the critical C:N or C:P ratios of detritus and microbes and the TERs of Gammarus spp.

Fatty acids (e.g., polyunsaturated fatty acids (PUFAs) and highly unsaturated fatty acids (HUFAs)) are critical biological compounds in aquatic food webs [58, 59]. Some fatty acids are critical for growth and reproduction while others are thought to maintain membrane fluidity at low temperatures [59]. However, little is known about the fatty acid requirements for Gammarus spp. in lakes and streams. Gammarus spp., like other invertebrates, have fatty acid requirements that must be filled through their diet as evidence for synthesis de novo has not been found. Future research should address the trophic transfer of essential fatty acids from microbes to Gammarus amphipods, as this research could make important contributions to Gammarus-microbe food web ecology and to our understanding of the microbial loop.

Acknowledgments

The author wishes to thank Frank M. Wilhelm and Chau D. Tran for insightful comments on an earlier version of this paper. In addition, this paper was greatly improved by comments from an anonymous reviewer.

References


Research Article

Dietary Preference of *Gammarus pulex* and *Asellus aquaticus* during a Laboratory Breeding Programme for Ecotoxicological Studies

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Received 29 March 2011; Accepted 1 June 2011

Academic Editor: Chris Lloyd Mills

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An investigation was undertaken to establish if *Gammarus pulex* and *Asellus aquaticus* preferred a diet of unconditioned, artificially or naturally conditioned alder leaves (*Alnus glutinosa*). Standardised, 24 hour *ex situ* feeding assays were undertaken with both species to determine their food preference. The results showed that *A. aquaticus* ate more leaf material compared to *G. pulex* (Z 23.909, \(P < 0.001\)) when exposed to all three test variables. Also, both *G. pulex* and *A. aquaticus* demonstrated a preference for naturally conditioned leaves compared to the other two variables, with unconditioned leaves proving the least popular food option for both macroinvertebrates (Z 18.803, \(P < 0.001\)). However, both species ate varying amounts of all the leaf treatments (Z 136.399, \(P < 0.001\)). Subsequently, the author outlined a feeding methodology for natural alder leaf conditioning that could be used during a laboratory breeding programme.

1. Introduction

What is the best diet for *Gammarus pulex* and *Asellus aquaticus* during a laboratory breeding programme and/or ecotoxicological study? Over the years, scientists have used a variety of nutritional supplements to feed macroinvertebrates during breeding programmes and experiments, including dog food [1], baby, and fish food [2]. If the macroinvertebrates were being bred for ecotoxicological studies (or as test subjects within bioassays) they need to be representative of wild specimens, and it is well documented that a test, animals response could be affected by their past history, diet, life stage, disease and so forth [3, 4]. Therefore, by feeding the animals with an unnatural diet, which may not contain the appropriate nutritional requirements, they could display a false negative/positive response during a test. Most workers, however, have gone down the more traditional route of using detritus to feed detritivores [4].

The role of allochthonous organic matter (e.g., leaves, wood) in streams and rivers has been extensively documented [5]. Freshly fallen leaves and other plant detritus that enter the water are rapidly colonized by microorganisms, a process referred to as conditioning [6]. There is considerable experimental evidence that shredders fed on detritus show preferences for and survive better on substrata that has been previously colonized by fungi, for example, Bueler [7]. It has been assumed that microbial colonization improves the nutritional quality of detritus through fungi having a differential ability to eliminate plant allelochemicals [8], fungal synthesis of micronutrients, production of mycotoxins [9], and/or the ability of detritivores to utilize acquired fungal enzymes [10]. Graca et al. [9] also demonstrated that *G. pulex* and *A. aquaticus* both discriminated between fungal mycelia and either fungally colonized or uncolonized leaf material. However, whereas *A. aquaticus* fed by scraping the leaf surface, thereby, selectively ingesting fungal mycelia, *G. pulex* nibbled the leaf, consuming both fungal and leaf matrix.

The food quality of detritus has been defined in terms of chemical (e.g., nitrogen and lignin), physical (e.g., resistance), and biological (e.g., microbial biomass) parameters. High-quality food has a low C:N ratio, low lignin content,
low resistance, and high microbial biomass [10]; therefore, alder would be described as a high-quality food. When *G. pulex* have been offered the choice between alder (*Alnus glutinosa*), beech (*Fagus sylvatica*), oak (*Quercus robur*), elm (*Ulmus glabra*), ash (*Fraxinus excelsior*), and willow (*Salix caprea*), the alder leaves were ingested at a much faster rate [11].

In contrast, Willoughby and Sutcliffe [1] found that the best diet for *G. pulex* was a mixture of conditioned elm and oak leaves. On this diet, the animals had a growth rate of approximately 150 µg day⁻¹ at 10°C in specimens of less than 16 mg body weight. In larger specimens, the rate apparently increased to about 350 µg day⁻¹. However, workers such as Nilsson [11] found that, at 15°C, an average of 1928.7 calories were produced from alder leaves g⁻¹ day⁻¹, which is considerably greater than other leaves, for example, beech (197.6 calories were produced from beech leaves g⁻¹ day⁻¹). The growth rate for Nilsson's smaller *G. pulex* specimens, which were fed on alder leaves was similar to the rate of 130.8 µg day⁻¹ at 15°C obtained by Willoughby and Sutcliffe [1] with a diet of oak and elm.

Researchers have previously used artificial [12] and natural [4, 13] methods to condition leaf material. The aim of this paper is to establish if the macroinvertebrates *G. pulex* and *A. aquaticus* prefer a diet of artificially or naturally conditioned alder leaves by undertaking ex situ feeding assays. In addition, the animals preference for conditioned and unconditioned leaf material will be assessed.

### 2. Materials and Methods

The *G. pulex* and *A. aquaticus* used in this study were obtained from a standardised laboratory breeding programme. The breeding programme's founder population originated from an unpolluted river source. Animals were captured, transported to the laboratory, and maintained under standardised conditions. The specimens were allowed to randomly copulate and the subsequent F₁, F₂, F₃ generations, and so forth were used for experimental purposes [4].

*G. pulex* (12–15 mg dry mass) and *A. aquaticus* (7–10 mg dry mass) males were used in the experiments. 24 hours prior to the test, 300 *A. aquaticus* were removed from the culturing tank and divided equally between 30, 500 mL sterile plastic pots (with screw lids), which contained 500 mL of deionised water. The animals were maintained under oxygen-depleting conditions without nutritional supplements at 15°C. For 16 hours per day, the animals were illuminated with a fluorescent light (with a specification for freshwater invertebrates), to simulate on a small scale the macroinvertebrates natural climatic conditions. The glow mimicked the thermal warmth and daytime illumination obtained from the sun radiation. The same procedure was also undertaken with 300 *G. pulex*. Bloor et al. [3] previously showed that in a deionised water test media (without aeration) both *G. pulex* and *A. aquaticus* could survive for several weeks without mortalities.

Alder leaves (*Alnus glutinosa*) were collected during the autumn fall (from Hillier's Arboretum, Romsey, UK), air dried, and stored in refuge bags (in a dry location) until required. The leaf material was cut into 1800 squares (length 2.0 cm and width 2.0 cm). 600 squares (1.16 g) were placed in 500 mL of enriched water (Table 1), inoculated with a standard amount of *Cladosporium* fungus (fungi:leaves, 1:20) and incubated for 10 days [12]. 600 squares (1.16 g) were soaked in 500 mL of river water containing 0.50 g of decaying detritus for 10 days (river water and detritus were collected from the River Itchen, Southampton, UK). The remaining squares were saturated in 500 mL of deionised water for 10 days.

The 1800 squares were then air dried for 24 hours and weighed. 120 squares from each treatment were put into the separate 500 mL test pots containing *A. aquaticus* and fed to the animals (the deionised water was not changed, and aeration was not applied); therefore, each test was replicated 10 times. After 24 hours, the squares were removed, air dried (for 24 hours), and reweighed. The amount of consumed detritus was then calculated by subtracting the final leaf weight from the conditioned weight. The same investigation was then repeated with *G. pulex*.

The data was analysed using PASW 18 statistical software. Initially, the Kolmogorov-Smirnov test was used to determine normality (*P* > 0.05). As the data was normally distributed, a parametric paired *t*-test was applied to establish if there was a significant difference between the initial and final weights of the leaves (*P* < 0.05). Finally, a general linear model was undertaken to investigate which leaf type was preferred by *G. pulex* and *A. aquaticus*.

### 3. Results

Application of Kolmogorov-Smirnov test indicated that there was no departure from normal distribution (*P* > 0.05) for the *G. pulex* unconditioned leaves (Z 0.160, *P* 0.757), natural conditioned leaves (Z 0.211, *P* 0.385), or artificially conditioned leaves (Z 0.151, *P* 0.411). The *A. aquaticus* data was also normally distributed (unconditioned leaves (Z 0.195, *P* 0.574), natural conditioned leaves (Z 0.163, *P* 0.621), or artificially conditioned leaves (Z 0.184, *P* 0.199)).

This enabled application of the parametric paired *t*-test (*P* < 0.05), which showed that there was a significant difference between the initial and final weight of unconditioned leaves (Z 8.157, *P* < 0.001), natural conditioned leaves (Z 34.259, *P* < 0.001), and artificially conditioned leaves (Z 9.918, *P* < 0.001) for *G. pulex* and also *A. aquaticus* (unconditioned leaves (Z 11.420, *P* < 0.001), natural conditioned leaves (Z 66.002, *P* 0.001), and artificially conditioned leaves (Z 35.146, *P* < 0.001)).

### Table 1: “Enriched” water recipe. 5 mL of each stock solution was mixed and made up to one litre with deionised water (extracted from [12]).

<table>
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Finally, a general linear model demonstrated that there was a significant difference between the amount of leaf material consumed by *G. pulex* and *A. aquaticus* (*Z* 23.909, *P* < 0.001), the type of leaf treatment consumed (*Z* 18.803, *P* < 0.001), and the amount each species consumed of each leaf type (*Z* 136.399, *P* < 0.001). Figures 1 and 2 illustrate that *G. pulex* and *A. aquaticus* consumed the leaf material in the order of naturally conditioned > artificially conditioned > unconditioned.

### 4. Discussion

Bacteria and fungi are important components of the detritivore diet [1], *G. pulex* and *A. aquaticus* both discriminated between fungal mycelia and either fungally colonized or uncolonized leaf material [9], which was illustrated by this study. The results clearly demonstrate that both species of macroinvertebrates preferred a diet of conditioned leaf material over unconditioned leaves, with natural conditioning being the favoured conditioning option. On comparing the initial and final weights of the natural and artificially conditioned leaf material, it can be concluded that natural conditioning produced heavier and noticeably softer leaves, which could be attributed to the colonization of microorganisms. Research has demonstrated that *A. aquaticus* feed, by scraping the leaf surface, thereby, selectively ingesting fungal mycelia, which would explain why these animals preferred the naturally conditioned leaves [9]. In contrast, *G. pulex* nibbles the leaf, consuming both fungal and leaf matrix [9]. As such, it might have been expected that the
G. pulex would not discriminate between the natural and artificial leaves, but the results of this study showed that natural conditioning was the diet choice for both species.

Studies have shown that G. pulex and A. aquaticus would grow to sexual maturity and reproduce on a diet of decaying leaves [4]. Few studies have measured or compared the rates of growth on different diets, but some authors have claimed that decaying leaves with rich flora of bacteria and fungi are more palatable and support faster growth of G. pulex than leaves without microorganisms [14].

However, workers, such as Graca et al., [9] demonstrated that although the growth of A. aquaticus was reduced when unconditioned leaves were provided, leaf conditioning does not influence G. pulex growth. This is because G. pulex has the ability to compensate for a low-energy uptake by reducing its energy expenditure. The mechanism behind this principle remains unclear but is probably linked to a decline in activity [15]. Whatever the mechanism, the outcome of this difference in response is that reduction in food quality has a greater impact on the energy balance of A. aquaticus than that of G. pulex, resulting in less energy being available. G. pulex may also resort to cannibalism in experimental situations when insufficient/inappropriate nutritional supplements are available [16], which could hinder a laboratory breeding programme.

When establishing a laboratory breeding programme for ecotoxicological studies, it is important that the animals are maintained in standardised and repeatable conditions. The animals need to remain stress-free or their toxicological response could be manipulated [4]. The animals diet is an important factor in maintaining a healthy and stress-free population, and consequently, it is important to keep the animals in the most natural environment as possible. By providing a diet that mimics their natural food source and contains the appropriate nutritional requirements for growth and reproduction, the animals would be representative of wild stocks during ecotoxicological studies. Therefore, the author would suggest that naturally conditioned alder leaves are an excellent diet choice for G. pulex and A. aquaticus populations within a laboratory breeding programme. The presented research supports the use of the feeding methodology outlined in Bloor [4], in order to breed and maintain healthy populations of both macroinvertebrates during a breeding programme.

Bloor [4] discussed that abscised alder leaves (Alnus glutinosa) should be collected during the autumn fall (from one tree), air dried, and stored. As such, the food source would be standardised as all the leaves were collected from the same tree on the same day. 10 L of river water and a handful of organic detritus should be collected from an unpolluted source and transferred to the laboratory in a lidded plastic container. On return to the laboratory, the water and detritus should be poured into a 15 L plastic box (the box should not be sealed with a lid). Handfuls of the precollected alder leaves should be submerged in the water and mixed with the precollected organic detritus (no precise measurements), which would inoculate the alder leaves with bacteria and fungus. The leaves should be conditioned for at least 10 days. After that time and when required, leaves should be extracted from the box and placed in the aquariums (excess liquid should be squeezed from the leaves to reduce the level of organic enrichment applied to the water). Additional air-dried leaves should then be immersed in the conditioning box to replace the utilised ones.

The leaves should be liberally scattered in the culture and rearing aquariums, to fulfil the animals nutritional requirements and replaced at regular intervals (enough leaves to cover the aquarium floor to a depth of approximately 50 mm). The juveniles should, however, be supplied with conditioned alder leaves for shelter and grazing but also fed upon adult faeces that should be syringed from the culture aquariums (when required), until the animals can feed entirely upon conditioned leaves (after about 25 days).

5. Conclusions

In summary, when undertaking a laboratory breeding programme with G. pulex and A. aquaticus, naturally conditioned alder leaves would be the recommended food source. As such, a feeding methodology was outlined that could be utilised during a breeding programme. The author would recommend that a priority for future research would be to investigate if the diet/health of laboratory populations of G. pulex and A. aquaticus could be improved by feeding a mixed diet.

References


Research Article

GamTox: A Low-Cost Multimetric Ecotoxicity Test with Gammarus spp. for in and ex situ Application

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Received 4 February 2011; Accepted 9 April 2011

1. Introduction

Gammarus spp. contain a few hundred freshwater, brackish, and marine species in the Northern hemisphere [1]. They stand for important keystone species in aquatic ecosystems, both functionally and structurally, due to their high abundance and biomass [2, 3]. As shredders and detritus feeders, gammarids participate in the detritus cycle and microbial loop [4]. Moreover, they show a wide foraging plasticity, being also herbivores, predators, and even cannibals. This flexibility might contribute to their ecological success in colonizing and invading ecosystems [2, 5] next to their high mobility (migration, drift), as well as their high reproductive capacity with several broods per female and year and the high number of offspring combined with a relatively long longevity (1-2 yrs).

Gammarids are sensitive towards different types of aquatic pollution [6]. Compared to Daphnia magna, gammarids are often more sensitive to metals or different types of pesticides, such as neurotoxic substances and especially pyrethroids (Table 1). In 17 from 57 studies gammarids were much more sensitive than Daphnia magna. As toxicity studies are difficult to compare due to different exposure times and test designs, a safety factor of 2 was applied to extrapolate from an exposure time of 48 h in the Daphnia magna studies to 96 h in the Gammarus spp. studies, as usually the LC_{50}s decrease with increasing exposure time, esp. during the first 48 h [7]. After application of a correction factor of 2 to the Daphnia magna studies, sensitivity shifted towards Daphnia magna in some cases. As gammarids spend extended periods of time in close contact with bottom sediments and in the water/sediment contact zone, they are standard test species in ecotoxicity testing in the USA and Europe for testing acute toxicity of sediments ((mortality within 96 h), OPPTS 850.1020 guideline) [8]. There are many ecotoxicological studies and several tests based on different measurement parameters have been developed so far, some of them have successfully been applied for both in situ and ex situ tests; however, all these isolated scientific efforts have never been collated and combined to one multimetric multilevel test, such as the proposal of GamTox as presented here.

Behavioral parameters in ecotoxicology studies are characterised by short response times, sensitivity, ecological relevance, and noninvasiveness, allowing for repeated measures and time-dependent data analysis [9, 10]. Changes in behavior may be used as important indicators for ecosystem health, because they rest on biochemical processes, but also reflect the fitness of the individual organism as well as potential consequences on the population level, such as
Table 1: Comparison of sensitivity of *Daphnia magna* and *Gammarus* spp. towards different types of chemicals. Daph-48: LC$_{50}$-48 h for *Daphnia magna*; Gam-96: LC$_{50}$-96 h for *Gammarus* spp. Daph/G: ratio LC$_{50}$ *Daphnia*/LC$_{50}$ *Gammarus*, where *Daphnia* proved more sensitive, that is, ratio below 1. D/Gam: ratio LC$_{50}$ *Daphnia*/LC$_{50}$ *Gammarus*, where *Gammarus* proved more sensitive, that is, above 1. Daph-96(2): LC$_{50}$ *Daphnia* 48 h/2 for correction of increase in toxicity with increasing exposure time. D/G corr: corrected ratio *Daphnia*/*Gammarus*.

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<th>Substance (µg/l)</th>
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Behavioral responses seem to be of similar sensitivity and efficiency as biochemical and physiological responses [10] and can be recorded both automatically and quantitatively, thus allowing the field of behavioral ecotoxicology to expand [12, 13]. Behavioral alterations have been linked to changes in acetylcholinesterase activity under exposure to neurotoxic pesticides in several aquatic species including gammarids [10, 14, 15].

Feeding rate is a sensitive sublethal endpoint compared to community-related measures which require changes in species composition before an impact is detected [16, 17]. Bloor and Banks [18] compared in situ and ex situ feeding assays with both the pollution-sensitive G. pulex, and the pollution tolerant A. aquaticus. Both, mortalities and feeding rates followed similar trends during the in situ and ex situ tests, but the response of test animals was amplified during in situ testing. Maltby et al. [17] found a strong positive correlation between in situ feeding rate of gammarids and macroinvertebrate diversity and a biotic index. Thus, the new GamTox might fill an important gap in current water quality assessment as it represents a measure for xenobiotic stress (“ecotoxicological” water quality assessment), which has neither been covered by existing biological water quality assessment methods (biodiversity, biotic indices) nor by chemical assessment methods.

The aim of this study was to develop a low-cost test protocol for a multimetric sublethal toxicity test with Gammarus spp. based on feeding activity, behavior and survival as well as biochemical biomarkers for both in and ex situ application to be included in routine water quality monitoring programmes.

2. Methods

2.1. Origin of the Test Organisms and Culture. Gammarus spp. can be collected in a reference stream, being rather un-
Gammarids and 2 elder leaves. The test chambers can be connected in the sediment and on the banks. Each chamber contains 10 of the stream in flow direction, attached with ropes to steel poles testing. laboratory test conditions for at least one week prior to conditions. Field-taken organisms need to be acclimated to water authorities for the choice of appropriate reference figures (e.g., [19–21]). Successful cultures have been described in several previous aquaria with unfiltered stream water for several months. maintained during several weeks or even cultured in aerated circulation and removal of high loads of particles. Every week the aquarium within a net cage (0.1 mm mesh size) for water pump with filter (Eheim aquaball 2400, 45 L) is placed in different protocols for culturing, the following procedure has been used successfully. Ca. 100 Gammarus spp. of mixed gender and size classes are collected from the site of origin. An aquarium from the field, and placed in a 20 L glass aquarium with a layer of 2 cm sediment (sand and pebbles) including organic matter (leaf packs) from the site of origin. An aquarium filter is cleaned and 50% of the water in the aquarium is exchanged by adding new stream water after acclimation to room temperature (between 16 and 22 °C). Once a week, 10 leaves of elder are added, twice a week the animals are fed additionally with frozen chironomids at libitum. The culture is kept for several months at room temperature in a dark room with daylight neon illumination with a photoperiod of 16-hour light/8 h night. Once a month the animals are checked visually, precopula pairs and small juveniles have been observed frequently, a sign of a healthy culture. Each time animals are taken out for experiments, new animals from the reference stream are added to refresh the gene pool of the culture.

2.2. Experimental Design Ex Situ. The experiments are executed under the same conditions as chosen for the culture concerning light and temperature (15–22 °C). The exposures are arranged in rectangular white-opaque hard-polyethylene containers (PE, 400 mL: 10 cm × 10 cm × 6 cm in high); the exposure lasts for 12 days without any renewal of water, food, or faeces (Figure 1). In each container 5 animals are placed and 3 replicates per treatment are set up, this test design represents the minimum set up concerning statistical evaluation. The PE containers contain either only water for tests with surface and waste waters or water and sediment (300 mL water, 100 mL sediment) for tests with toxic sediments from, for example, waste disposals and overlaying water. The water in each container is continuously aerated through a pipette to reach 100% oxygen saturation. One preconditioned elder leaf is added to each container as both food source and substrate. Every 4 days survival, behavior (either: response to prodding with a forceps, or: recording in the Multispecies Freshwater Biomonitor) and feeding rate (0, 25, 50, 75, 100% of leaf consumption) is monitored. Leaf consumption is visually monitored either by eye or by photographs in an easy manner as the animals produce feeding traces, they scelitize the leaf. The containers are covered with a PE lid to avoid evaporation. Control survival and feeding rates of the animals of >80% after 12 days of exposure in the reference water are regarded as quality criteria for the experimental design.

2.3. Experimental Design In Situ. In situ exposures are arranged in test chambers of the Multispecies Freshwater Biomonitor (MFB) [12, 22] (Figure 2). Eight acrylic glass cylinders of 15 cm length and 5 cm inner diameter capped with screw rings on both ends, which contain a nylon net of 0.5 mm mesh size are exposed in the current flow and attached both in the sediment and on the banks using stainless steel poles. Each test chamber contains 10 animals and 2 preconditioned elder leaves. Every 4 days survival and behaviour of the animals are recorded with the Multispecies Freshwater Biomonitor for 30 minutes (generating 3 subsequent online measurements) operated by a car battery [23, 24]. In these conditions the animals can be monitored in their exposure cages in the stream without disturbance at several subsequent occasions until the end of the experiments after 12 days. At the end of the experiment the animals are manually collected and counted, the feeding rate is noted. With this setup, experiments can be performed at low and normal water levels in small wadeable streams with permanent flow. In case of rapidly increasing water levels due to heavy rainfall events and floods, the equipment has to be checked more frequently in the field.

2.4. Multispecies Freshwater Biomonitor (MFB). The described experiments can be executed in the test chambers, with or without the MFB, however, without the MFB; the animals need to be checked manually every 4 days in the cages, and this might create additional stress for the animals. The MFB has already been used in several in situ applications. Several ex situ and in situ tests have been conducted with the fully automated real-time-based Multispecies Freshwater Biomonitor, developed by Gerhardt et al. [22]. The system is based on quadrupole impedance conversion technique that simultaneously records several behavioral parameters of a wide range of aquatic organisms, for example, different
crustaceans, insect larvae, oligochaets, molluscs, tadpoles, and several fish species. During exposure, the organisms move freely between two pairs of electrodes on each sidewall of a test chamber, which receives unfiltered stream water or exposure water [22]. The organism’s behavior is expressed as movements that lead to changes in an electrical field and these are measured as changes in the impedance of the system. For example, (1) locomotion: swimming and crawling results in irregular amplitudes and frequencies, (2) resting: small signals that cannot be separated from background noise, (3) ventilation: regular, high-frequency movements with, for example, pleopods to establish a constant water flow across the gills, and (4) feeding: species-specific patterns for grazing, filtering, and hunting. The impedance converter proved to be a sensitive and quantitative tool for use in behavioral, ecological and ecotoxicological studies, which makes it a promising tool for continuous biomonitoring purposes [25], as proven with *Gammarus* spp. exposed to a copper pulse [12] or in river monitoring stations along the rivers Meuse (NL), Aller (GER) and Rhine (F) [10].

### 3. Results

The new GamTox test protocol was validated in laboratory exposures with different types of polluted surface water [26]: (1) leakage from the waste disposal containing solvents, caused increased mortality and decreased feeding activity of the gammarids. The stream water showed elevated levels of iron and Ammonium and the biological water quality class was described as unsatisfactory (according to the European WFD class 4). (2) the effluent from a municipal waste water treatment plant containing pesticides (WWTPs) caused high mortality and decreased feeding activity of *Gammarus fossarum* as well as >20% AChE inhibition, a toxicity threshold previously defined [14, 15, 27].

GamTox *in situ* validation was conducted in a small reference stream with locally abundant *Gammarus* spp. Five tubes with each 10 organisms and two elder leaves were exposed *in situ* for 12 days. After 12 days survival and feeding rates of the gammarids resident in the stream were >85% in all chambers.

### 4. Discussion

The GamTox test protocol proved to be easy and sensitive towards chloroalkanes, aromatic compounds, and pesticides in the laboratory assays regarding survival and sublethal test parameters such as feeding behavior and AChE inhibition. A first test of the protocol *in situ* showed the practicability of the test in a reference stream, further test validation in streams polluted by pesticide pulses are currently being performed and will be published separately.

In previous tests with *Gammarus fossarum* studying survival and behavior, however, with different exposure times, *Gammarus fossarum* proved more sensitive towards AgCl2-exposure (0.7 mg/L) than other test species (*Pseudokirchneriella subcapitata, Vibrio fisheri*) and especially locomotory activity recorded in the Multispecies Freshwater Biomonitor revealed effects already after a few hours of exposure at concentration levels which proved to be lethal after 7 days of exposure [28]. Sediment pore water from a solid pulp waste disposal was tested with different standard toxicity tests and compared with *Gammarus fossarum* acute toxicity test (24-hour survival, feeding activity). The waste disposal was polluted with PCBs, PAHs, and Cd. While no effects were seen in algae (*P. subcapitata*) growth and photosynthesis,
Gammarus fossarum showed effects already after 24 hours of exposure with increasing locomotion, a sign of avoidance behavior. Chronic population-relevant effects of this disposal could also been seen after 8 days (Ceriodaphnia dubia: reproduction), after 10 days (Chironomus riparius: survival) and after completed emergence of Chironomus riparius [29]. This indicates, that Gammarus’ behavior can be used as an early warning test for ecologically relevant evaluation of both acute and chronic toxicity of even other freshwater invertebrate species. This validation was the base to continue with simplifying and automation of toxicity testing with Gammarus, as it is described here in the new test protocol for both in and ex situ testing. This new test protocol needs further validation by the water authorities performing water quality bioassessment in their routine monitoring programmes.

The following new concept for an ecotoxicological water quality assessment as part of a taid-based integrated water quality monitoring is now proposed (Figure 3). In a literature study [27] it could be shown that Gammarus spp. react in a very sensitive way towards pesticide pulses, esp. neurotoxic insecticides, and locomotory behavior (drift, locomotion) seems to be the most appropriate test parameter. In some small streams polluted temporarily by pesticide pulses, gammarids were reported missing or their population densities were decreasing which was observed during several years of routine biomonitoring. As gammarids are reported indicators for the saprophyte class 2 (beta-mesosapro), corresponding to “good” water quality according to the EU-WFD, the decline or presence/absence of gammarids could be a first indicator of pesticide pollution. This evaluation can be done by just re-evaluating existing bioassessment data over the past times. Additionally, the SPEAR index (species at risk) can be calculated from macrozoobenthos data (http://www.systemecology.eu/SPEAR/index.php). Like this, problem sites might be discerned where GamTox as described above can easily be conducted in or ex situ, without the need of a sophisticated laboratory, as both the toxicity test and the culture can be managed at room temperature and no expensive equipment is required for the test. GamTox aims to verify the observed stress (e.g., Gammarus presence/absence, macrobenthos, SPEAR, and chemistry) as being based on ecotoxicological effects. Additionally, manpower can be reduced by executing GamTox in a fully automated manner in the MFB. In case GamTox (survival, feeding, and locomotion) indicates toxicity further tests such as specific biomarkers and/or reproduction might be carried out if bio/chemical water analyses reveal elevated concentration levels of pesticides and/or surpass the proposed environmental quality standards.

Acknowledgments

Olivier Geffard (Cemagref, Lyon, France) is gratefully acknowledged for performing the AChE analysis on gammarids. AquaPlus (Zug, Switzerland) is thanked for financial support of parts of the experimental work.

References


Research Article

Watercress and Water Quality: The Effect of Phenethyl Isothiocyanate on the Mating Behaviour of Gammarus pulex

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Received 23 December 2010; Accepted 8 March 2011

Academic Editor: Michelle Bloor

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Watercress releases phenethyl isothiocyanate (PEITC) upon wounding as a defence against herbivores. PEITC levels released from watercress farms are elevated due to cropping, washing, and processing and are thought to lead to adverse effects on Gammarus pulex in chalk streams. This study elucidates the sublethal effect of PEITC on reproductive behaviour of G. pulex, employing ex situ tests to investigate the disruption of precopular pairing under conditions simulating in situ exposure. Mean time to separation of precopular pairs was 89 ± 6 minutes for watercress wash water (1 g watercress per litre water) and 81 ± 15 minutes for pure PEITC (1 µL/L). Re-exposure to watercress wash water to simulate the pulsed operation at a watercress farm did not alter behavioural response. The repeated interruption of reproductive behaviour under in situ conditions would impair long-term reproductive success and could explain in part low abundance of G. pulex downstream of watercress farms.

1. Introduction

England has the principal resource of chalk streams and rivers in Europe, many of which are designated conservation sites (e.g., Sites of Special Scientific Interest and Special Areas of Conservation) [1]. Chalk streams support an abundant and diverse macroinvertebrate community with many specialised and rare species, for example, the fine-lined mussel, Pisidium tenuilineatum, and the mayfly Paraleptophlebia wemeri [2]. Gammarus spp. have been identified as keystone species [3] with the potential to exert disproportionately powerful effects on the community structure and ecosystem processes. Gammaridae, which usually exist in chalk streams in very large numbers, are the principal detritivore and dominate the prey assemblage. As generalists, they are found along the length of the river.

Although chalk streams favour a rich and diverse ecology [1], their use by humans imposes a risk to some biota. Chalk headwaters provide an ideal location for cultivation of watercress (Nasturtium officinale) as the nutrient content is naturally high and the constant temperature of aquifer-derived water provides protection from winter frosts and promotes vegetation growth during the colder months [4]. Whilst watercress occurs naturally as a common macrophyte in most reaches of chalk streams and can dominate during the summer period [5], it has the capacity to release substances that exert toxicological impacts on freshwater organisms. In particular, watercress is a source of phenethyl isothiocyanate (PEITC), a compound derived from the catabolism of glucosinolates present in cell vacuoles within tissues of plants containing them [6]. Glucosinolates occur naturally in watercress and other Brassicaceae, many of which are important economic food crops. The primary hydrolysis product of the glucosinolate present in greatest quantities in watercress (i.e., 2-phenethyl glucosinolate, also known as gluconasturtiin) is 2-phenethyl isothiocyanate (PEITC). A number of studies identify the role of the degradation products in the defence of the plant against herbivorous insects [7–9]; freshwater systems possess few specialist herbivores, and chemical feeding deterrents provide the most effective means of protection against generalist herbivores [10, 11].

Watercress is chemically defended from generalist herbivory by the glucosinolate-myrosinase system and the
PEITC thereby released upon attack. Isothiocyanates, in particular PEITC, released by watercress have well-documented allelopathic and genotoxic properties [11] and have a role in the plant defence against herbivorous macroinvertebrates such as snails, caddis flies, and gammarids [12]. Few studies, however, have elucidated the effect of isothiocyanates on aquatic macroinvertebrates. The effectiveness of PEITC as a feeding deterrent has been established [13], and behavioural tests [8, 13, 14] have shown avoidance of watercress by Gammarus pulex.

Despite these known impacts of PEITC on G. pulex, both watercress and G. pulex are considered characteristic of chalk streams [1] and are often observed at high abundance [4]. In some cases, however, low numbers of G. pulex have been linked to watercress farming and processing operations. For example, a reach of the Bourne Rivulet (Hampshire, UK; a tributary of the River Test) is entirely maintained by waters discharged from a watercress farm and washing and packing operation. Only watercress is grown at this site, but the washing and packing operation handles a variety of salad leaf products, including other Brassicaceae. Bruising, maceration, and wounding of watercress and other produce during harvesting and processing triggers the glucosinolate-myrosinase system, thereby increasing PEITC levels in the discharge waters. In this specific case, the observed reduction in G. pulex numbers and species diversity has been of concern due to the status of the watercourse as a chalk stream headwater which has an important role in the functioning of the River Test ecosystem downstream [15]. Measurement of PEITC from an aqueous matrix is, however, problematic and no standard methodology has been established.

For this particular location, the depleted abundance of G. pulex [15] may be due to the stream containing elevated concentrations of PEITC as a result of the entire stream being maintained by waters used in the watercress farm and washing and packing operation. There is clearly a need to improve our understanding of the causes of low G. pulex numbers in streams affected by watercress production and processing, such that effective mitigation measures may be informed by a quantitative scientific evidence base. We suggest that the low abundance of G. pulex could be explained by two ecotoxicological responses to PEITC. First, there may be an acute effect whereby mortality is substantial but incomplete. Second, there may be a sublethal effect whereby reproduction is impaired. Acute and sublethal effects are not mutually exclusive; this study forms part of a series of works and will focus on sublethal effects on reproduction in this instance.

G. pulex undertake a period of mate-guarding behaviour prior to mating. An adult male takes hold of a female and the pair remains together in precopular position for a few days until the female moults. Mating then occurs before her cuticle hardens, and the fertilised eggs are laid into a brood pouch. The female becomes attractive to males again at, or slightly before, the hatching of the eggs [16]. Precopular pairing may be affected by exposure to toxicants and separation has been reported as a sensitive and rapid indicator of stress due to cadmium [17] and raised ammonia levels [18]. Precopular separation along with neonatal and population growth are reported as consistently sensitive endpoints in an evaluation of methods used to evaluate toxicity to freshwater ecosystems [19]. The reproductive behaviour test has also been used to determine the effects of vertebrate-type endocrine disrupting chemicals; there is also evidence to support the use of pheromonal control of mating in G. pulex [20].

This overall aim of this study, therefore, is to determine the extent of the effects of PEITC on precopular pairing of G. pulex. We note that in situ exposure of G. pulex to substances derived from the watercress farm and processing operation occurs on a pulsed basis. The large area of watercress cropping beds and the crop washing facility on-site operates primarily on an "office hours" basis leading to potentially elevated levels of PEITC arising on a 24-hour cycle. Specifically, this study aims to (1) quantify the effects of exposure to PEITC on separation of precopular G. pulex pairs, (2) determine the effects of re-exposure on precopular separation, and (3) assess whether exposure impairs reformation of precopular pairs.

2. Materials and Methods

2.1. Preparation of Test Solutions. A single batch of mature (i.e., ready for harvest) watercress was washed briefly to remove coarse debris. Care was taken to minimise handling damage to avoid subsequent loss of PEITC from the sample. The sample was then frozen to store for tests and prevent further hydrolysis of glucosinolate to PEITC. A single batch was used to minimise variability in the glucosinolate concentration in crops grown under different conditions [21, 22]. Media (dilution) water was prepared by vigorously aerating tap water for more than two hours to remove chlorine. Frozen watercress leaves and stems (large stems were excluded) were weighed and added to a measured volume of media water. The media water/leaf mixture was stirred once, that is, a stirring rod making one revolution of the beaker and then leaf and stem debris was filtered out using a 250 μm mesh. The resulting wash water was used as the test solution. It was assumed that the freezing process had caused complete lysis of cell walls and thus complete and immediate hydrolysis of glucosinolate to PEITC. Analytical grade PEITC was used to prepare PEITC test solutions. PEITC is heat and moisture sensitive [23] and required dilution with analytical grade methanol in a stock solution. Test solutions were made on the day of the test (1 μL/L) by dilution of the stock with aerated media water.

2.2. Test Organisms. G. pulex were collected from the River Meon at Funtley Mill, Hampshire (NGR SU556089). They were acclimatised to laboratory conditions in a constant temperature room at 14 ± 2°C, with a photoperiod of 8 hours daylight and 16 hours darkness under cool white fluorescent tubes (mean bench-top illumination of 800 lux), in glass tanks with tap water media which had been vigorously aerated for more than two hours to remove all chlorine. They were fed a diet of alder leaves (Alnus glutinosa (L.)) presoaked in river water, and 10% (by volume) media changes were made every two days for a period of two weeks.
The breeding population was then maintained under these conditions. Precopulatory pairs were used for sublethal tests as the interruption of reproductive behaviour would be indicative of an unsustainable population. The use of sublethal data would also provide a greater level of sensitivity and in applying the results to the process at the study site would afford a greater degree of protection within the receiving water. Initial trials resulted in immediate separation of control precopulatory pairs due to handling stress when they were transferred to and from the holding vessel media; several methods employ physical stimulation as a technique to isolate males from females [24–26]. Of a number of different methods examined for the transfer of precopulatory pairs (e.g., use of a wide bore pipette, a sieve, a spoon, or emptying out media), the advantage due to minimising handling stress was compromised by other factors such as the time taken or the potential dilution of the test solution by media water. The use of the wide bore pipette was thus chosen, providing minimal handling stress and transfer of media water but not impractically prolonging the transfer of organisms to the test solution.

2.3. Quality Control. Tests were carried out as far as possible according to quality control methods prescribed by laboratory standard ISO 17025 [27]. Daily temperature checks were carried out to ensure the constant temperature room remained within an acceptable temperature range. Equipment was calibrated using United Kingdom Accreditation Service (UKAS) approved methods (e.g., balance, Finn-pipettes, water quality meters, and timers). Calibrated volumetric glassware was used. Solvent controls (analytical grade methanol diluted to the same concentration as in the PEITC test solution with aerated media water) and media controls were carried out for tests using PEITC test solutions and media controls carried out for tests using watercress wash water test solutions. All control organisms were subject to the same handling as the test organisms. Water quality validation criteria for dissolved oxygen (>60% ASV), pH (constant to within 0.5 unit), conductivity (<10% change) were also assessed for each test. No adjustment or correction of test solutions was required as validity criteria were met on all occasions.

2.4. Two-Hour Time to Pair-Separation Test. As part of their mating behavior, G. pullex form precopulatory pairs, separating once fertilisation has taken place [20, 28]. Initial observations of precopulatory pairs in wash water were made with a view to carrying out the precopulatory separation (GaPPs) test described by Pascoe et al. [28]. This procedure exposes pairs to the test solution for one hour followed by an enforced separation and records the time taken for pairs to reform. However, watercress wash water caused pair separation during the one-hour exposure and a variation of this method was used in which precopulatory pairs were exposed to a single dose of watercress wash water for a two-hour period. The concentration of watercress wash water test solution selected was guided by the ratio of leaf to water washed in the salad washing and processing factory, which washes at a ratio of 1 g leaf to 50 mL water [29]. Isothiocyanate-producing crops make up approximately 50% of product washed; accordingly, a concentration equivalent to 1 g watercress per 100 mL wash water was used. The endpoint used was time to separation of pairs and was recorded at 15-minute intervals. Glass crystallising dishes covered with a watch glass were used as the test vessel, with 150 mL of test solution and 5 precopulatory pairs added to each test vessel. At least 4 (and up to 8) replicate vessels were employed, depending on the number of pairs available.

2.5. Precopulatory Re-Exposure Test. A series of re-exposure tests was also conducted to elucidate responses of precopulatory pairs to pulsed exposures as experienced in the Bourne Rivulet. The wash process at the farm operates daily (0730 to 1700 h weekdays and 0630 to 1600 h weekends) and outside this the discharge consists of borehole water from watercress bed flow only. Consequently, there is a period every 24 hours when there may be very low levels of PEITC present in the discharge. During the processing hours, the wash lines are changed at frequent intervals, for example, on 10 June 2008, there were 43 different product lines washed and packaged, and each product contained a varying proportion of watercress in the total weight washed [30]. The nature of the discharge from the processing operation is thus highly variable. Re-exposures were carried out in a laboratory simulation of the temporally variable nature of the wash and process factory water, but variation of the crop processed was not simulated. At the end of the two-hour precopulatory separation test, the test organisms were removed to clean water and left to re-pair over a period of 48 hrs. The re-paired organisms were then re-exposed to fresh test solution as per the first test.

3. Results

3.1. Sublethal Tests. Four tests were carried out with watercress wash water as the test solution. Test organisms from two of these were re-exposed to freshly prepared watercress wash water, one at test end plus 24 hours and the other at test end plus 48 hours. Similarly, four tests were carried out using a PEITC test solution and test organisms from two of these were re-exposed, one at test end plus 24 hours and the other at test end plus 48 hours. Both the watercress wash water and the PEITC solution disrupted reproductive behaviour (Figure 1). The ET_{50} (i.e., the exposure duration at which 50% of precopulatory pairs had their natural behaviour disturbed and separated) was calculated by hypothesis testing for each test using ToxCalc v5.0.32 environmental toxicity data analysis software [31]. Calculated ET_{50} values (Table 1) varied markedly between tests, but with considerable overlap of the ranges for tests using watercress wash water and a solution of PEITC, as also demonstrated by separation patterns over the whole two-hour period (Figure 1).

3.2. Re-Exposures. On re-exposure to freshly prepared watercress wash water and PEITC solution at the same concentration as the first exposure, pair separation was observed in a
Figure 1: Mean cumulative proportion of pairs separated (±SE). Precopular pairs were exposed at Time = 0, the mean of 4 tests over the course of the 2 h exposure is shown for each test substance (carried out on separate occasions, with a total of 119 pairs exposed to wash water, 105 pairs exposed to PEITC and the control (mean of 8 tests, with a total of 157 pairs exposed). Vertical bars show standard error; n denotes the number of tests. There was immediate separation of at least one pair in all the PEITC test solutions (i.e., by the first 15-minute reading). There was separation of at least one pair in all the watercress wash water test solutions after 45 minutes. There was a steady increase in the number of pairs separated over the course of the two-hour test to 70% or greater in all wash water test solutions (maximum 95%, mean 84%) at the test end. The pattern of response for the PEITC solution was very similar (maximum 100%, mean 85%) at test end.

Table 1: Summary of ET50 values. The proportional data were arcsine square root transformed and the ET50 calculated using maximum-likelihood probit or logit analysis. WW1–WW5: samples of watercress wash water. P1–P5: samples of PEITC solution.

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<tr>
<td>WW2</td>
<td>106*</td>
<td>103–110</td>
</tr>
<tr>
<td>WW3</td>
<td>89</td>
<td>78–102</td>
</tr>
<tr>
<td>WW5</td>
<td>84</td>
<td>72–93</td>
</tr>
<tr>
<td>P1</td>
<td>48</td>
<td>38–56</td>
</tr>
<tr>
<td>P2</td>
<td>119</td>
<td>108–133</td>
</tr>
<tr>
<td>P3</td>
<td>85</td>
<td>77–92</td>
</tr>
<tr>
<td>P5</td>
<td>40</td>
<td>20–56</td>
</tr>
</tbody>
</table>

*Calculated using logit model—all others with probit.

Similar manner as for the first exposure; however, it occurred sooner (Figures 2 and 3) and resulted in an overall greater proportion separation after two hours (Figure 4) although there was no statistically significant difference. The ET50 (95% CI) values for pairs re-exposed to watercress wash water were 87 (77–106) and 41 (27–51) minutes. The ET50 (95% CI) values for pairs re-exposed to PEITC solution were 54 (41–64) and 40 (19–53) minutes. For all re-exposures, the ET50 was reduced, that is, pair separation occurred sooner (Figure 5), although it should be noted that as only two re-exposures were carried out, a statistically robust assessment of the variability could not be made. The rate of pairs reforming was assessed for organisms returned to clean water at the initial test end (Figure 6). In all instances except one (Test WW3), where the proportion of pairs re-forming was recorded, the number of pairs was greater after a period in clean water than at the end of the test exposure. After return to clean water, there was generally a proportion of G. pulex that did not re-pair and the mean control pair re-formation achieved was 75% (n = 6). However, on a single occasion (test WW5), control re-formation was 100%; the two control pairs that had separated during the initial test were able to reform in the following 24-hour period.

3.3. PEITC Concentration in Wash Water. Although analysis of the test solutions for PEITC was not carried out, GC-MS analyses of watercress wash water may give an indication of the amount of PEITC that the test organisms were exposed to. Dixon [32] estimated the amount of PEITC released by weight as a mean value of 529 ± 45 µg/g leaf washed. Therefore, precopular pairs were exposed to PEITC at an estimated concentration of 5.3 ± 0.5 mg/L PEITC.
4. Discussion

4.1. Sensitivity of Gammarus pulex to PEITC and Watercress Wash Water. The use of Gammarus spp. for ecotoxicological testing at both acute and sublethal levels of sensitivity has been well documented and evaluated [33–35] and includes the specific use of a precopular separation test [28]. Protocols for acute testing with *G. fasciatus*, *G. pseudolimnaeus*, and *G. lacustris* are available within the United States Environmental Protection Association test methods collection [36]. Johnson et al. [37] recognised the importance of appropriate bioassay choice, design, and quality assurance/quality control measures in effluent assessment and control. The choice of *G. pulex* as the test organism in this study was influenced by the observed impact on Gammaridae recorded in the receiving water downstream of the watercress farm and processing operation at the study site.

Application of sublethal tests as employed in the present study is influenced by the nature of PEITC. This compound has an unknown degradation pathway in watercress wash water, which may depend on temperature, pH [38], and/or the presence of other members of the family Cruciferae [39] and the volatility of glucosinolate breakdown products [6]. The volatility of PEITC constrains longer-term tests; a continual dosing system would not have been practicable for this study. It was possible to achieve a precopular separation endpoint over a short period of exposure to wash water solution and the response was also recorded throughout the duration of the two-hour exposure period. This response was similar for the watercress wash water solution and the re-exposed organisms, and the PEITC solution although for the re-exposures occurred sooner.

The mode of action of PEITC from watercress wash water has not yet been established, although many studies have documented the relationship between terrestrial herbivorous...
invertebrates and glucosinolate producing crops [40–43] and the use of chemoreceptors in adaptive behaviour. Watercress wash water has elicited a response in adults [40–44], feeding adults [8], and reproductive adults (this study). Therefore, although the ingestion of PEITC may cause an acute response, it is possible that detection of PEITC by chemoreceptors or its metabolism within cells may also be eliciting the sublethal behaviour that has been recorded. In studies relating to the use of PEITC as an anticarcinogen, which have investigated and quantified the uptake of isothiocyanates, rapid cellular uptake has been demonstrated [45–47]. Rapid cellular uptake would concur with the sublethal response by reproductive G. pulex, seen within 2 hours, and their subsequent recovery. We therefore speculate that the mode of toxic action of PEITC on Gammarus spp. is probably initially at a cellular level. PEITC may additionally act separately via ingestion [8] with long-term exposure (exposure possibly via several pathways) leading to mortality.

The effect of re-exposing precopular pairs to watercress wash water and PEITC solution was analysed using four different methods. The graphical comparison of rates of separation during the two-hour exposure (Figures 2 and 3) illustrated that the effect was seen more quickly in organisms already exposed to the toxicant. This result was supported with lower ET50 values for exposures to both watercress wash water and PEITC than for the initial exposures, that is, the effect would be seen in half of the population more quickly than for the first exposure. The two-hour proportion separated showed that the overall sensitivity of the pre-exposed organisms was however not significantly increased.

4.2. Practical Implications. Exposure to watercress wash water and PEITC produced a behavioural response measurable in reproductive adults. The behavioural response seen in reproductive adults was carried out at a single concentration and it is not clear from this work whether fluctuations in their response would be altered by a change in the dose regimen. It is possible that with an increase in the exposure duration and/or if the dose was increased beyond a certain level, the separation of reproductive pairs may give way to a toxic response leading to adult mortality. There will, therefore, be implications for the sustainability or survival of populations of G. pulex in the receiving water below watercress farm discharges where exposure to PEITC leads to similar doses to those used in this study.

The reversibility of the behavioural response may also depend on the exposure duration and dose. Returning organisms to fresh water at test end allowed the interrupted reproductive behaviour to recommence; at the dose tested, the separation was due to a transient effect. However, it is important to note that the opportunity for male G. pulex to fertilise females is limited to a few hours after the female molts [16]. The mate guarding behaviour thus ensures access to the female when she is receptive. In relation to operations at the study site, the repeated disruption by daily pulses of discharge of watercress wash water would reduce the opportunities for males to fertilise and therefore, over a long period, reduce the reproductive success of the population.

Analysis of the number of pairs re-forming showed that there was an inconsistent increase in pair re-forming over a 48-hour period, and even in controls, 100% re-pairing was not generally achievable. The number of pairs re-forming was also subject to the natural pattern of the reproductive cycle [16], and thus a proportion would naturally separate anyway. It is interesting to note that separation of re-exposed pairs (Figures 2 and 3) occurred sooner in the tests which were carried out after 24 h rather than 48 h in clean water, even though this was not reflected in an overall greater proportion separation at the end of the two-hour period or a much lower ET50.

Where low diversity or low abundance is noted in the macroinvertebrate populations of chalk stream receiving waters below watercress farms, the potential effects due to PEITC should be considered. Watercress producers are required to meet consent conditions for a variety of water quality parameters such as suspended solid load and biological oxygen demand (BOD). The contribution of PEITC-induced effects should also be examined.

4.3. Wash Water Sample Preparation. The method of preparation of watercress wash water test solution was based on the salad wash process at the study site. Sources of variation were minimised, particularly where nominal concentrations
were prepared for the acute test using very small quantities of leaf only. Wash water prepared using larger quantities of both leaf and stem may have introduced variability due to the potential for different glucosinolate content in each part of the plant. Although a comparison of PEITC present in stem and leaves has not been made, Gil and Macleod [39] showed that there were different levels of PEITC produced from N. officinale seeds and leaves. Likewise, Rosa [48] described significant variation between glucosinolates present in the roots and aerial parts of Brassica seedlings. Newman et al. [14] also reported that toxicity of frozen watercress roots to G. pseudolinnaeus was similar to the leaves.

4.4. Further Work. It was only possible to carry out re-exposure tests when there were sufficient precopulatory pairs after the return of test organisms to clean water. The use of much larger numbers of pairs in the initial tests would have resulted in more pairs becoming available for re-exposure. However, this was governed on a practical basis by the facilities and human resources available for test setup. Similarly, a longer time period in clean water may have increased the numbers of pairs available for re-exposure. A compromise was made between practicability and relevance to field simulation at the farm where re-exposures occur within 24 hours. Re-exposure tests were carried out where at least 3 replicates of 3 pairs were possible as well as control replicates, although this was less than recommended by standardised acute test methodology such as Environment Agency [49] acute single concentration Daphnia magna test where 6 replicates and 20 organisms are prescribed. It should be recognised that the use of a larger number of pairs would have increased the statistical robustness of the method.

Further testing with freshly collected samples of salad wash water, taken directly from the wash lines at the study site, would provide a direct link to the crop washing process and its effect on gammarids in the receiving water. It would additionally be beneficial to increase the number of sublethal tests carried out with PEITC solution and watercress wash water to assess the level of variability in the G. pulex response and confirm the reproducibility of the test. The reliability of the short-term sublethal test could also be evaluated by further tests to establish the natural background variability against which the stress-induced precopulatory separation can be measured [33]. An estimate of the unimpaired re-pairing rate for the population could be made by artificially separating control organisms prior to a period in clean water.

5. Conclusions

The secondary metabolite PEITC produced by harvested and processed watercress has a sublethal effect on G. pulex breeding pairs. The effect is evident at concentrations anticipated to be produced by the leaf washing process at the study site. Re-exposures of G. pulex precopular pairs to PEITC in watercress leaf wash water did not illicit a significantly different separation response although all re-exposures had a lower ET50 and responded more quickly during the exposure. The organisms did not appear to acclimatise to PEITC or become less able to withstand its effect. Further tests and re-exposures would establish if this was a consistent finding. The adaption and extension of a more commonly used reproductive pair separation methodology (i.e., to re-expose organisms to freshly prepared test solution) reflected more accurately the exposure pattern experienced by organisms in the receiving environment. This novel use was considered important to the relevance of the particular situation in the receiving water below the discharge from the study site. The mode of action of the toxicant has not been confirmed, although behavioural effects are evident. The similar response seen in both PEITC solution and watercress leaf wash water solution would indicate that PEITC is the causative agent.

Acknowledgment

The authors gratefully acknowledge the financial support of this study by the Vitacress Conservation Trust.

References


Research Article

Imaging and Documenting Gammarideans

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Received 29 March 2011; Revised 30 May 2011; Accepted 30 May 2011

Academic Editor: Almut Gerhardt

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We give an overview of available techniques for imaging and documenting applied to gammarideans and discuss their advantages and disadvantages. Although recent techniques, such as confocal laser scanning microscopy (cLSM), focused ion beam scanning electron microscopy (FIB SEM), or computed microtomography (μCT), provide new possibilities to detect and document structures, these high-tech devices are expensive, and access to them is often limited. Alternatively, there are many possibilities to enhance the capabilities of established techniques such as macrophotography and light microscopy. We discuss improvements of the illumination with polarized light and the possibilities of utilizing the autofluorescence of animals such as the gammarideans. In addition, we present software-based enhancing tools such as image fusion and image stitching.

1. Introduction

Imaging and documenting specimens is an important part of the basic biological investigations, particularly of morphological and taxonomic work. Informative images are also required for oral or poster presentations. In recent years, new documentation techniques for zoomorphological investigations have been developed. Studying the morphology appears to have become an increasingly “high-tech” field of science, demanding complex machines and fast computers with complicated software packages. Despite this, new ideas involving facile and inexpensive methods and free computer software capable of running on older computers have become available and can improve classical morphological approaches significantly. Notable among these new high-tech methods are tools for three-dimensional documentation, such as different types of computed tomography (CT) (e.g., [1]) and confocal laser scanning microscopy (cLSM) (e.g., [2, 3]). The new tools for enhancing more classical methods are mainly based on computer algorithms, such as image fusion or image stitching (e.g., [4, 5]), in addition to 3D approaches such as structure from motion (e.g., [6, 7]). Furthermore, the combination of different methods and also the adoption of techniques from one field into another have yielded promising results.

New techniques can also offer new insights. Former complex preparation processes that were necessary to answer certain questions may become superfluous (at least in some cases). They have been superseded by new methods yielding comparable results which may be faster and/or do not require the destruction of rare specimens. Examples of such cases are computer tomography substituting serial sectioning [8] and fluorescence microscopy being used instead of alizarin staining [9].

The crustacean taxon Gammaridea with almost 6,000 living species must be considered a well-investigated group, at least taxonomically. Despite this, many morphological details of the numerous described species remain significantly understudied, and many structures are still waiting to be discovered and understood (e.g., [10, 11]). This is despite such detailed morphological studies being the basis for investigations of ecological, phylogeographical, or evolutionary aspects of the species being studied. Conclusions about the ecology of these animals, for example, on the ecological interplay of native and neozoic animals, are tightly coupled to the understanding of the detailed morphology of the species in focus [12, 13].

For the investigations of morphological aspects of gammarideans, newly emerged techniques yield new opportunities. Therefore, we tested a large variety of up-to-date
documentary techniques, compared them against one another, and considered the advantages and disadvantages of each technique with a special focus on the cost-benefit equation. Tested techniques ranged from computer microtomography to different types of fluorescence microscopy to enhanced variants of white-light microscopy and macrophotography. We aimed at offering an overview of available techniques applicable to facilitate and improve future studies on gammarideans.

2. Different Methods and Discussion

2.1. General Remarks. Specimens of the following gammaridean species were the basis of the present investigation: *Gammarus roeselii* Gervais, 1835, *Dikerogammarus villosus* Sowinsky, 1894, *Dikerogammarus haemobaphes* (Eichwald, 1841), and *Orchestia cavimana* Heller, 1865. In general different types of software were used to optimize the recorded images. To overcome limitations of depth of field of an image, several images of the same image detail were recorded in different focus levels. These images form a so-called image stack, with one single image referred to as “frame”. Frames of a stack were fused with freely available image fusion software (CombineZM, CombineZP, Image Analyzer) to one sharp image. To overcome limitations of field of view, several images were combined to one panorama with image stitching software (Adobe Photoshop CS3 “Photomerge” function or the freely available Microsoft Image Composite Editor). Adobe Photoshop CS3 or Gimp was also used to optimize contrast and brightness. In slightly blurred images, “mask unsharp” filters were used to improve these.

2.2. Documenting Entire Specimens with Macrophotography

2.2.1. Methods. A live specimen of *Gammarus roeselii* was photographed with a Panasonic FZ-50 digital camera, equipped with an additional Raynox M-150 macroscopic lens. Two synchronized Nikon SB-20 flashlights were used for illumination.

Complete female specimens of *Dikerogammarus villosus*, which had been stored in 70% alcohol, were documented with macrophotography in their storage liquid. They were placed in front of black velvet background and photographed with a Canon macrophoto lens EF-S 60 mm on a Canon EOS 450D digital camera as a stack of frames under different settings. (1) Under “normal” light. Two Leica KL 1500 cold-light sources were used to illuminate the specimen, with the angle of light at about 45° to minimize reflections. (2) Under crossed polarized light [14]. A polarizing filter was not only mounted on the camera lens but also on two cold-light sources. Filters on the two cold-light sources were adjusted parallel to each other, and the filter on the camera lens was adjusted with perpendicular filter direction. (3) Fluorescence settings [9]. Cold-light sources were equipped with cyan filters and the camera lens with a red filter. (4) A stereo image under polarized light. In the settings 1–3, stacks were recorded and fused (see above).

2.2.2. Documenting Gammarideans Alive or Dead. Due to the fast movements of their limbs, especially of the continuously beating pleopods, the entire living gammarideans can only be documented by a single image, best illuminated using a flashlight (Figure 1). The advantage of such images is the retention of the natural color and appearance of the specimen on an image and the possibility to depict the specimen in its natural surrounding. However, such images usually suffer from two difficulties. First, often not all structures of the specimen are entirely sharp, because the depth of field is limited. The second difficulty is that reflections often occur.

With dead or anesthetized specimens, the first problem can be overcome by recording several frames of the same image detail in different focal layers and by fusing the resulting stack of frames by the application of a computer software for image fusion into one sharp image of high depth of field. Such programs are freely downloadable from the internet today and are usually easy to handle. With a camera equipped with a macro lens, it is often sufficient to take two or three frames in a stack to obtain a sharp final image. Image fusion programs also compensate size or shift differences while refocusing, usually occurring when using macro lenses and also stereo microscopes. More problematic are rotations, but even this issue can be overcome if recognized later. Yet if it is recognized right after recording, we recommend just a rererecording of the stack, because this is often less time-consuming and more accurate. For macrophotography, the camera should be mounted on a tripod or a repro stand. With the latter, it is possible to change the distance between the lens and the object in small steps, without rotating or moving the camera in the other two dimensions.

For illumination, flashlights or cold-light lamps can be used. With cold-light lamps, it is possible to adjust the camera and control settings in live preview. Many modern digital cameras are supplied with remote software so that it is possible to control the camera directly on the computer and view a live preview on the screen.

2.2.3. Getting Rid of Reflections: Polarized Light. An important shortcoming of photographing stored specimens is...
Figure 2: Comparison of different methods applied to an entire specimen of *Dikerogammarus villosus* stored in alcohol. All images are fused from several focal planes. (a)–(c) Macrophotographs. (a) “Normal” macrograph, with optimized uniform light. Bubbles are evident on the black velvet, which was used as background. Due to reflections, individual elements of the limbs are difficult to distinguish (arrow). (b) Macrograph under polarized light. Bubbles are not apparent. Borders between individual elements of the limbs more conspicuous (arrow). (c) Macrofluorescence. Borders between individual limb elements are well apparent due to the color contrast (arrow). (d) Composite fluorescence image [4]. Details well contrasted, also the individual limb elements (arrow).

Reflections caused by the storage liquid. Even if the specimen is removed from its liquid, the wet surface produces a lot of reflections. Taking the specimen out may also cause the damage of the specimen as it may desiccate; at least, setae may easily agglutinate. Photographing wet specimens “wet-dry” is especially not recommended for rare material. Reflecting the specimen immersed in a sufficient amount of their storage liquid moves the surface reflection away from the specimen, although this usually does not completely erase all reflections (Figure 2(a)).

An easy solution to get rid of reflections is the application of crossed polarizing filters. Polarizing filter foils are cheap and can be easily attached to the light sources. One after the other is then turned until maximum elimination of reflections is achieved. This can be best tested by adjusting the filters while observing a piece of polished metal, for example, pincers or needles. When all filters on the light sources are adjusted parallel, the filter on the camera lens is turned until maximum elimination of reflections is achieved, adjusted with perpendicular filter direction. When using black velvet as a background polarizing filters make especially the small bubbles invisible that usually cover at least in some areas of the submerged velvet (cf. Figure 2(a) versus 2(b)). For the best results and undisturbed metering, the filter mounted on the camera lens should not be a piece of polarizing filter foil, but a commercial circular polarizing filter.

Applying polarized light has further advantages. It is well known that storing specimens in alcohol or formalin is not the best preservative for color pattern, yet it is often the only option. Therefore, most museum specimens and especially old-type specimens (if still available) show significantly less contrast than living or freshly killed specimens, as the original color has faded away. Yet, the investigation of types is still an important aspect in modern science. Applying crossed polarized light filters improves the contrast between parts of the specimen significantly. In our case, the borders of the podomeres of the trunk limbs, which are not apparent under normal light conditions, become marked under crossed polarized light settings (cf. Figure 2(a) versus 2(b)).

2.2.4. Enhancing the Contrast: Use of Fluorescence. Even stronger contrast than that with crossed polarizing filters can be achieved by the application of fluorescence. Fluorescence photography can be done using a camera or a camera in combination with a fluorescence microscope. In the two cases, the optics are much different. In the case of macrofluorescence, that is, with a camera equipped with a macro lens, the contrast stems from significant differences in the color between heavily sclerotized or calcified areas, which appear in our case orange to pink, while membranous areas remain white (Figure 2(c)). The contrast of the borders of the podomeres is significantly higher than with normal white-light and still better than with crossed polarized light settings.

Using microscopes other than stereo microscopes for documenting the entire specimens is usually limited to very small specimens, as such optical devices have an extremely
limited depth of field and field of view. With the software-enhancing tools of image fusion and image stitching, it becomes possible to overcome these shortcomings and document also larger specimens on a microscope. This is especially interesting for applying fluorescence, as the fluorescence light sources on a microscope usually provide a more focused and more uniform light. Since the light produced is monochrome, fluorescence microscopes are generally equipped with b/w (monochrome) cameras (see below), which cannot detect color differences. Therefore, fluorescence microscopic images provide differences in brightness between sclerotized/calcified and membranous areas. In the present case, the results are comparable to the macrofluorescence image, based on the recognizability of the podomeres. Yet the fluorescence microscopic image (Figure 2(d), see method below) has a significantly higher resolution than the macrophotographic image (Figure 2(c)), as it is a composite image. This advantage is unfortunately paid by a significantly longer time to produce the image.

While fluorescence has significant advantages for photographing colorless specimens, polarized light settings are superior for photographing freshly killed specimens which still possess their original coloration, as this cannot be well documented with fluorescence imaging. Furthermore, to apply macrophotography, it is necessary to have a completely darkened room, otherwise the white-light from the surroundings will conceal the fluorescence. If such settings are not available, crossed polarized light settings should be chosen. Fluorescence microscopy should only be used to document the entire specimens if they are sufficiently small, or further details are needed. In other cases, the method is simply too time-consuming. For important type specimens, it might still be the method of choice to obtain high-resolution images for having “back-up” information. Such high-resolution images could be treated as “virtual specimens”.

2.2.5. Additional 3D Information: Stereo Images. Stereo images of the entire specimens can also be used to enhance certain morphological details, which would not be visible on a normal macrophotographic image of a not well-contrasted specimen (Figure 3(a)). Spatial arrangements of the legs are well depicted with such a method. The approach to present stereo images to improve the understandability for the reader is also helpful for tomographic data. Different projections can be used for emphasizing different structures in such images. Volume-rendering settings give a good access to structures of the outer surface of the scanned specimen (Figure 3(b), cf. Figure 5(c)). Although in our case the result of the scan is not too highly resolved (see method below), modern micro-CT scanners provide resolutions comparable to overview SEM images (although not comparable in the resolution of higher magnified details, see below). Maximum intensity projections (MIPs) appear more similar to certain light microscopical techniques (Figure 3(c)). It gives the object a more transparent appearance providing access to inner structures, while still showing outer structures. The advantage of these two projection methods is that they can be set up fast, just through adjusting brightness/contrast in the projection. More time-consuming is the visualization as a so-called surface model. In this case, it is usually necessary to mark structures by hand, called “segmentation”, and this procedure is, therefore, used for emphasizing chosen structures (see below).

2.3. Documenting Details: Light Microscopy and Comparisons with Alizarin Stainings

2.3.1. White-Light Microscopy. A mandible of Dikerogammarus haemobaphes was isolated and embedded in glycerol. The distal part of the palp of the mandible was documented in a Zeiss Axioskop using transmitted light under bright field and dark field settings. A Bresser MikroCam directly mounted on the C-mount of the microscope was used for photography. The focal plane was shifted manually, and a stack of frames was recorded. The obtained stack was fused with the software Image Analyzer. Based on the bright field stack, a 3D surface model was generated using the macros of
2.3.2. Fluorescence Microscopy. A complete specimen of Dikerogammarus villosus (the same specimen as for macrophotographic settings 1–3) was documented using fluorescence microscopy. Only the autofluorescence of the specimen was utilized, and no staining was applied. The specimen was kept in a small petri dish with ethanol and placed on a Zeiss Axio Scope 2 with a 1.25× objective, equipped with a b/w AxioCam. Several stacks were recorded and processed following the composite image principle (fusion and stitching, see above). The embedded Dikerogammarus haemobaphes mandible used for white-light microscopy was also documented using the Axio Scope 2 with a 10x objective under UV light. In this case, the AxioCam was not used, but an external DCM 510 ocular camera capable of recording live color [9]. Here, in addition, a stack was recorded and fused, but with a sub-optimal result. The distal part of the mandible was, therefore, additionally recorded on a Zeiss Observer equipped with a spinning disc and an AxioCam. This stack was three-dimensionally projected with the freely available software OsiriX. From this, a stereo image of a maximum intensity projection (MIP) of this stack was exported.

2.3.3. Alizarin Staining. Another mandible of a specimen of Dikerogammarus haemobaphes was treated after the protocol of Brösing et al. [16] for alizarin staining. This includes macerating the specimen in 10% KOH at 100°C for one hour, then adding a small amount of alizarin red directly into the solution. After 10–15 minutes, the specimen is removed and rinsed with demineralized water. Afterwards the specimen is stored in 70% ethanol. Calcified parts manifest red-violet, other structures, especially membranous areas, become transparent. The specimen treated in this way was placed in 70% ethanol in a petri dish. Reflected light was applied, and a stack of frames was recorded with a Canon macrophoto lens MP-E 65 mm on a Canon EOS 450 D digital camera.

2.3.4. Comparing the Results of Light Microscopy and Alizarin Staining. The possibility to overcome almost any limitation of a restricted depth of field or a limited field of view is a major breakthrough. Although recording stacks is more time-consuming than recording single images, the benefit is overwhelming, especially for high magnification. Here, single images (frames) have an extremely limited depth of field, which is nicely demonstrated by the single image of the mandibular palp tip (Figure 4(a)). Therefore, applying image fusion and also stitching should be seen as mandatory (Figures 4(b) and 4(c)) leading to the complete mandibular palp tip being in focus, including the protruding setae. The technique can be applied to both bright field (Figure 4(b)) and dark field microscopy (Figure 4(c)) and in principle to other contrasting settings. The choice of the proper contrasting method can give access to different structures or at least emphasize different structures. The advantage of bright field microscopy is that the original color of the investigated specimen is evident, depending, of course, if the specimen still has some of its original coloration or if this has already faded. Contrasting settings, such as dark field microscopy, can give access to structures almost invisible in bright field and, furthermore, emphasize thin structures but with the disadvantage of losing the original color information.

Recording stacks has another advantage. The limited depth of field provides information about the three-dimensional arrangement of structures, a fact that can be used in different ways to depict three-dimensional arrangements. A fast developing application is the depth from defocus approach (e.g., [17, 18]). In this case, a surface model is created (Figure 4(d)), and the fused image based on the stack is rendered onto its surface (Figure 4(e)). The method is usually optimized for conditions in material sciences and industrial applications (e.g., [19]); therefore, there remain some disturbing artifacts for biological specimens, such as the bumpy background (Figures 4(d) and 4(e)). Yet this approach has potential and also demonstrates that it is, in principle, possible to extract 3D information from a white-light microscopy stack.

Other approaches were put forward by Kamenz et al. [20], which process the stack in a comparable way to surface models based on CT data sets (see below). The idea to process a stack of light microscopic images following the method of Haug et al. [15] was applied here to the mandibular palp tip (Figure 4(f)). Compared to the depth from defocus approach, color information is lost, although a much more naturally appearing 3D arrangement of the setae is obtained. It is important to note that all used software for this approach is open access and can be easily applied [15].

Compared to SEM images (cf. Figure 4(g)), most light microscopic images appear less sharp (cf. Figure 4(b)) and also cannot give as good access to the surface structures. A light microscopic technique for accessing surface information can be autofluorescence microscopy (see above for entire specimens). However, autofluorescence is not always coupled to the outer cuticle but can also give access to inner structures. In the present case, we investigated the specimens also using white-light settings. Interestingly, here, the autofluorescence is strongest on the setae. The specimen appears to be close to molting, as under the cuticle a second row of setae is visible. In addition to the rather sharp image detail, this is information that cannot be obtained by either SEM or white-light microscopy. Fluorescence microscopy is, therefore, seen as a technique yielding enormous potential.

Despite this, in cases where the object exhibits strong autofluorescence, this causes scattered light, and the fusion results are often unsatisfying. This is especially true for live color fluorescence microscopy (Figure 4(j)). Unfortunately, most methods to overcome scattered light are coupled to grayscale imaging, such as cLSM, spinning disk (Figure 4(h)), or apotome. Here, technical enhancements are
Figure 4: Different microscopical methods applied to structures of the mandible of *Dikerogammarus haemobaphes*. (a)–(h) Tip of the mandibular palp ((a)–(f) and (h) depict the same specimen). Red-cyan glasses needed to experience the stereo effect in (d)–(f) and (h). (a) A single focal plane of a bright field frame. Note the limited depth of field and the resulting blurring. (b) Fused image based on a stack of 20 frames of different focal planes, including also the frame from (a). (c) Fused image of a stack of 27 dark field frames. (d) Red-cyan stereo anaglyph of a surface model based on stack used for image (b). (e) (b) rendered onto (d). (f) Red-cyan stereo anaglyph based on the stack used for (c), following the procedure of Haug et al. [15]. (g) SEM micrograph of a comparable specimen. (h) Same specimen as in (a)–(f). Red-cyan stereo anaglyph based on an autofluorescence image from an inverted microscope with a spinning disc. The spinning disc suppresses scattered light. Due to the inversion, the other side of the palp is visible (note that the setae seem to come out of the image instead of reaching into it). Image flipped horizontally to enhance comparability. (i) Sagittal cut of the left part of the cephalothorax with left mandible, alizarin staining. Arrows point to the pivots of the joint. (j) Autofluorescence micrograph of a right mandible in median view. Arrow points to new pars incisiva underneath the current cuticle. Abbreviations: set: setae, pi: pars incisiva, pm: pars molaris.

also desirable. However, in this case, the fused images are still significantly better than single images.

2.3.5. Additional Information from Live Color Fluorescence. Live color imaging yields another source of data. For understanding the functional morphology of an arthropod, its pattern of sclerotization and, in the case of gammarideans, calcification is of interest. Calcification can well be accessed through staining KOH-macerated specimens with alizarin. We used the protocol provided by Brösing et al. [16] to stain a mandible (Figure 4(i)). Calcified areas appear in violet. In the case of the mandible, these are the tips of the pars incisiva, the pars molaris, as well as the pivot joints of the mandible. Autofluorescence with live color provides a comparable
image: pars incisiva and pars molaris have a more yellowish color while the remaining cuticle is more bluish under UV-fluorescence settings. Autofluorescence may, therefore, be a good alternative for macerating and staining specimens, especially where specimen numbers are limited. Because this is a mandible of the same individual from which the mandibular palp was depicted (Figures 4(a)–4(h)), the supposed close-to-molt situation can be observed. The new pars incisiva is visible under the old cuticle. Surprisingly, it is quite far away from the old structure. Fluorescence microscopy may be an interesting tool to investigate such close-to-molt situations in detail in the future.

2.4. Documenting Details: Micro-CT Scanning

2.4.1. Methods. A critical-point-dried specimen of *Gammarus roeselii* was documented using a Stratec Fanbeam μScope X-ray micro-CT scanner. The specimen was fixed with a piece of plasticine. The plasticine yielded a weaker contrast than the specimen but had to be virtually removed using the freely available software ImageJ. The produced stack was further processed in OsiriX. The stack was projected as a minimum intensity projection (MIP) and as a volume rendering. From the two projections, Stereo images of lateral views were exported. Two structures, the gut and the endopod of the right gnathopod, were marked by hand in several frames (about every tenth frame). Such a mark is termed a ROI (region of interest). The ROIs missing in the remaining frames were computer-generated using the automatic ROI volume function. The complete process is also termed “segmentation”. The segmented structures were highlighted in the MIPs. Additionally, surface models of these ROI volumes were produced and exported as object (.obj) files, an object file being a format for storing a description of the surface of a 3D object. The surface is described as triangles or higher-degree polygons. Again, a surface model of the entire animal was produced using a relatively high threshold and exported as an .obj file. These .obj files were imported into the freely available 3D software Blender and processed further here (adding color, smoothening, rendering, etc.).

2.4.2. Processing Micro-CT Data. A micro-CT data set can be well used to discuss the possibilities and shortcomings of the principle methodology. While fast projection methods have been discussed above for depicting entire specimens, we focus on the surface model. For extracting details from a CT data set, it is necessary to go through the process of segmentation (Figures 5(a) and 5(b)). In many cases, this needs to be done by hand, but in well-contrasted data sets, many structures can also be traced via a greyscale threshold. Furthermore, it is usually not necessary to mark the structures in each single frame. Yet, these automatically produced markings should be checked, as in some cases “weird” arrangements are produced with the automated function. Through this process a three-dimensional surface of the marked structure can be generated, which then is usually assigned a certain color.

In the present case, we marked the different podomeres of the endopod of a gnathopod, as well as the gut for orientation (Figure 5(c)). For even better orientation, the outer surface of the specimen was also rendered as a surface model choosing a low threshold giving the surface a skeletonized appearance but providing good reference points (Figure 5(d)). Again it must be mentioned that all the data processing was conducted in OsiriX. The resulting surface model was exported and further treated in the open source 3D software Blender. The fully segmented gnathopod endopod appears “shaved” (Figure 5(e)) compared to an SEM image of the same structure (Figure 5(f)), yet, all podomeres are represented as three-dimensional structures, allowing a proper visualization of the principle geometric shape of all elements. Such 3D models can be further used as a basis for biomechanical investigations.

2.5. Documenting Details: Scanning Electron Microscopy (SEM, FIB SEM)

2.5.1. Methods. A *Dikerogammarus haemobaphes* mandible and the second gnathopod of a *Gammarus roeselii* specimen were prepared for scanning electron microscopy for further comparisons. To remove debris from the cuticle, the specimens were rinsed in distilled water containing a detergent and sonicated for 20 seconds in a Merck Eurolab ultrasonic cleaner (as recommended by Felgenhauer [21]). Best results were obtained with a solution of a detergent for cleaning dental prostheses. After dehydration in an alcohol series, the specimens were critical-point-dried and sputter-coated with a gold palladium mixture. SEM work was done with a Zeiss DSM 962 scanning electron microscope. A second gnathopod of a specimen of *Orchestia cavimana* was investigated on a FEI Quanta 3D-FEG focused ion beam SEM (FIB SEM). This machine allows direct microdissection of the specimen while it is in the vacuum chamber of the FIB SEM. A sensillum was dissected in order to have access to the inner architecture of this structure. The digital images obtained from the SEM were trimmed in Adobe Photoshop CS3.

2.5.2. Access to Minute Details and Internal Structures with SEM and FIB SEM. Scanning electron microscopy is the method of choice for documenting surface structures when color does not matter. This technique enables magnifications far beyond the possibility of light microscopy, that is, up to 100,000 times magnification. However, in lower magnifications SEM also provides good results, for example, when whole limbs are documented. Although the depth of field of SEM is already much greater compared to light microscopy, it can also be extended by making stacks of frames of different focus planes and fusing them. The problem with SEM might be the accessibility of such machines and the fact that in most cases the specimen has to be dried and sputter-coated. Therefore, SEM should not be applied to rare specimens.

FIB SEM is a new technique that enhances the features of a conventional SEM, with the possibility to dissect minute structures right in the vacuum chamber of a SEM. Not only
parts of the specimen obscuring the structures of interest can be cut away but also parts of the body surface can be removed to uncover inner structures of the specimen. This is possible through the application of a Gallium ion beam, which can be used to “mill off” structures with an extreme preciseness and fine resolution. In the present case, we applied FIB SEM to investigate the inner structure of a sensillum on the dactylus of a second gnathopod (Figures 5(g)–5(i)). The area in which the milling process was applied was extremely small and could not have been prepared in a comparable way with another method. FIB SEM has a high potential for investigating such minute structures in detail directly after locating them on the specimen. This is regarded as a significant advantage compared to sectioning, which demands to locate the structure before sectioning, then embed and process the specimens and locate the structure of interest again. FIB SEM gives a much more direct access.

3. Conclusions

Our overview of different techniques for imaging and documenting gammarideans shows that besides high-tech methods such as FIB SEM and cLSM, there are also facile and inexpensive techniques, which can be applied to gammarideans and other arthropods. We discussed the advantages and disadvantages of the different methods. There is no universal method. The best to apply depends on the individual task. However, testing different methods offers the possibility to reveal new details or simply to get an informative picture of high quality. Apart from that science sometimes might just be beautiful.

Acknowledgments

This study would not have been possible without dear colleagues providing kind help and access to their facilities.
Many thanks to the team of the Central Facility for Electron Microscopy, especially Reinhard Weih, the Institute of Analytical and Bioanalytical Chemistry, Focused Ion Beam Center, especially Joel Pikarsky, Boris Mizaiikoff, and Christine Kranz, the Institute for Molecular Genetics and Cellular Biology, especially Thomas Gronemeyer, Nils John-son, and Judith Müller, Uwe Wolfram at the Institute of Orthopaedic Research and Biomechanics, and Stefan Liebau at the Institute for Anatomy and Cellular Biology, all from the University of Ulm. The authors also thank all people involved in programming freely available software. In the present study, these are particularly GIMP, Microsoft Image Composite Editor, CombineZM, CombineZP, Image Analyzer, Blender, OsiriX, and ImageJ. Special thanks to Calum MacNeill, Isle of Man Government Laboratory at Douglas, Isle of Man for improving the English of the paper. The authors thank one anonymous reviewer for helpful comments. J. T. Haug was kindly funded by the German Research Foundation (DFG) under Wa-754/15-1. This work was part of the project Wa-754/16-1 (DFG).

References


Research Article

Effects of Different Salinities on Juvenile Growth of Gammarus aequicauda (Malacostraca: Amphipoda)

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Received 8 March 2011; Accepted 14 April 2011

Academic Editor: Chris Lloyd Mills

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Gammarus aequicauda is a euryhaline amphipod that is a common inhabitant of brackish environments of the Mediterranean Sea. In the Ebro delta, the population density of G. aequicauda is highly variable throughout the year. The main objective of this study is to investigate the effect of salinity on the growth of G. aequicauda juveniles. G. aequicauda embryos and juveniles can survive and grow in the laboratory between 2 psu and 40 psu salinity, depending on the previous acclimation period for the reproductive individuals. Adults acclimated at 34 psu produced embryos and juveniles that survived and developed at salinities between 9 psu and 40 psu; adults acclimated at 9 psu produced embryos and juveniles that could develop in oligohaline conditions. The lower growth rate values were 10.9 \( \mu \text{m d}^{-1} \) and 13.5 \( \mu \text{m d}^{-1} \) at 40 psu and 2 psu, respectively, with the higher values of 18.0 \( \mu \text{m d}^{-1} \) and 18.5 \( \mu \text{m d}^{-1} \) at 19 and 34 psu, respectively.

1. Introduction

Salinity is one of the main environmental factors that exerts an intense pressure on crustaceans by controlling their distribution. Gammaridean amphipods living in coastal, estuarine, and adjacent freshwater environments tolerate highly variable salinities, including hypo-osmotic conditions [1–7]. Understanding the tolerance limits in the different life cycle phases of amphipods will be helpful in further studies on their life history and population distribution.

G. aequicauda (Martynov, 1931) is one of the most common and abundant amphipods from lagoons and brackish environments of the Mediterranean and the Black Sea [8–17]. G. aequicauda is a euryhaline species, being very resistant in isolate habitats from the sea with extensive ranges of salinity. This species has an important trophic role in the transport of energy to a higher consumer level, and its feeding activities contribute greatly to macrophyte fragmentation, thus enhancing microbial colonization and macrophyte decomposition [9–12, 18–22]. Although the distribution, life cycle, reproductive biology, and population dynamics of G. aequicauda have been studied in several coastal environments [8, 13, 14, 18, 22, 23], laboratory studies on the effect of salinity on survival and growth are scarce [24].

The Ebro delta is an estuarine environment that is influenced by rice crops. Agricultural practices regulate the hydrological cycles of the system, inducing periods of desalination and salination that are inverted in comparison with natural estuaries [25–27]. The Encanyissada lagoon is a shallow eutrophic coastal lagoon in the Natural Park on the right-hand semidelta of the Ebro delta [28]. The lagoon comprises a fluctuating ecosystem that receives fresh water drainage from irrigated lowland rice fields from April to October [25].

G. aequicauda is a characteristic and abundant macrofaunal species in the Ebro delta [9, 13]. G. aequicauda individuals are subject to large variations in salinity. For example, the salinity in Encanyissada lagoon fluctuates between 4 and 37 psu, with lower concentrations near the shore, where sudden drops of salinity down to 2-3 psu are observed during fresh water “discharge”. In this habitat, population densities of G. aequicauda vary greatly throughout the year [13], and the most important factors that regulate population density
of this amphipod are unknown. Prato et al. [24] showed that the survival of *G. aequicauda* was affected by salinity with the optimal range of 15 psu to 36 psu. Kevrekidis et al. [14] concluded that life history, growth, reproduction are not markedly affected by low salinity (0.3–5.7 psu) although low salinity does affect embryo viability. Previous studies on the biology and population dynamics in the Ebro delta showed that abundance is not correlated with changes in salinity [13]. Researchers argue about the ontogenetic variations in which embryos and juveniles can survive and develop are currently unknown.

The present work was aimed at studying the effects of salinity conditions on the survival and growth of juvenile *G. aequicauda* to provide information on their distribution range, growth, and ecology in the Ebro delta.

2. Materials and Methods

2.1. Collection and Acclimation of Amphipods. Amphipods were collected in February 2008 from Encanyissada lagoon at the communication channel of the lagoon with Alfacs Bay (Ebro Delta; 40°37’N 0°36’E) on the NW Mediterranean coast [13]. Collection was done with a hand-held net with a mesh size of 500 µm and a mouth aperture 35 cm in diameter. Water temperature at the collection site was 17°C, and salinity was 34 psu. Before experiments, animals were held in the laboratory for two days at this temperature and salinity.

After transferring the animals to the laboratory, the individuals were divided into two groups. Group 1: individuals were maintained in a 100 L aquarium provided with aeration and with natural sea water at the same conditions of the collection site (17°C and 34 psu salinity) and under an artificial 12:12 h light:dark cycle. Group 2: individuals were acclimated in a 100 L aquarium with a salinity of 9 psu stepwise to increasingly dilute media (decrements of ≤3 psu, at intervals of 1-2 days) about 2-3 weeks before any experiment was undertaken. The temperature and photoperiod were identical to that in group 1. The different experimental salinities were obtained by diluting filtered seawater from Alfacs Bay (34 psu) with appropriate quantities of freshwater (conductivity: 300 µS/cm). Hyperhaline conditions (40 to 50 psu) were obtained by adding artificial seawater at a salinity of 70 psu. Salinity was checked by a WTW InoLab Level 3 refractometer. Both groups were fed the macroalgae (Chlorophyta) *Ulva* sp. (in excess) obtained in the collection sites. Twenty percent of the water from the aquariums was renewed every 2-3 days at this temperature and salinity.

2.2. Experiments. To determine the effect of salinity on juvenile growth, brooding females from group 1 were directly transferred from water with the acclimation salinity (34 psu) to water with constant salinities of 0 (300 µS/cm), 2, 4, 9, 19, 34, 40, and 50 psu. The mean brood size of *G. aequicauda* from the Ebro delta populations was 23.6 [13]. Females with a brood size <15 were discarded. Three brooding females were placed individually in 10 L aquariums for each treatment (three replicate). After hatching, females were removed, and 15 recently hatched juveniles were maintained in each aquarium and were reared until the end of juvenile development. The experiments were conducted under a 12:12 h light:dark regime. Temperature was maintained at 17 ± 1°C (±SE), *Ulva* sp. were provided as food, and 50% of the water was exchanged every 48 hours. Five live juveniles from each treatment group and a replicate were measured cephalon length every 7 days for a total of 42 days.

To compare the effect of acclimation on juvenile growth at oligohaline conditions (<5 psu), brooding females of group 2 were directly transferred from the acclimation salinity (9 psu) to water with the following constant salinities: 0 (300 µS/cm), 2, 4, and 9 psu. The procedure was identical to the first experiment.

2.3. Measurements. Cephalon length (CL) was measured from the anterior margin (front) to the posterior dorsal margin of the cephalon. Body length (BL) was measured from the front to the base of the telson. CL was used as an individual size reference, because BL is difficult to measure, especially in live individuals; however, CL is an appropriate measure to estimate the size of the amphipods. To determine the relationship between BL and CL, 96 individuals were measured. The relationship between CL and BL was studied by a regression analysis. To measure CL during the experiments, each amphipod was placed on a glass slide and was examined for <1 min to minimize the effects of hypoxia and handling stress. Measurements were taken with an image analyzing system (AnalySIS, Münster, Germany) connected to a stereomicroscope (Nikon SMZ800).

2.4. Data Analysis. The relative growth of body parts was determined using the allometric equation $BL = aCL^b$. Using the transformed variables $\log_{10} BL$ and $\log_{10} CL$ (logarithmic equation): $\log_{10} BL = \log_{10} a + b \log_{10} CL$, tests for departures from isometry ($H_0$: $b = 1$) were performed on the slope values obtained by the Student’s t-test ($P < .001$). The statistical analysis of the growth data in the first and second experiments was performed by one-way ANOVA using the SigmaStat 3 (Systat Software Inc., USA) software package.

3. Results

3.1. Measurements. There exists a positive correlation between BL and CL ($r^2 = 0.9795$ and $n = 96$). The regression equation was:

$$\log_{10} BL = 1.211 \log_{10} CL + 0.2197.$$  \hspace{1cm} (1)

(See Figure 1).

The relationship between BL and CL shows positive allometric growth ($b = 1.211$; $H_0$: $b = 1; t = −16.23; P < .001$). Therefore, body length (BL) was between 5 and 7 times greater than cephalon length (CL), depending on the size of the amphipod.

3.2. Experiment 1. *G. aequicauda* embryos and juveniles from brooding females acclimated at 34 psu salinity can survive and grow at salinities between 9 psu and 40 psu
Gammarus aequicauda at different salinity conditions. Abbreviations: AC, acclimation conditions.

3.3. Experiment 2. G. aequicauda embryos and juveniles derived from females acclimated at 9 psu salinity can survive and grow in oligohaline conditions (2 psu and 4 psu) but not in freshwater (Table 1; Figure 2(b)). At 0 psu salinity, the ovigerous female died after 12–24 h, and no juveniles were observed. After 42 d, individuals reared at 2 psu salinity were smaller than those reared at 4 psu and 9 psu, but the differences were significant only for the 4 psu treatment group (P < .018, ANOVA). At 4 and 9 psu salinity conditions, precopula pairs were observed at the end of experiment (42 d). The growth rate values (CL) obtained were 13.5–17.3 \( \mu \text{m d}^{-1} \) (Figure 3).

4. Discussion

The relative growth of Gammarus aequicauda was previously studied by Kevrekidis et al. [33]. These authors showed that there is a positive correlation between cephalic length and body length. However, Kevrekidis and Lazaridou-Dimitriadou’s equation differs from the equation found in the present study in terms of the slope (\( b = 1.237 \) versus \( b = 1.211 \), resp.). Both equations give similar results only for small sizes. The differences may be due to the geographically variability in the allometric growth.

Salinity as an environmental factor has been considered mostly in terms of its effects on survival, distribution, and reproductive strategies in marine and brackish-water amphipods [1, 3, 34–36]. The determination of the potential capacities of a population in relation to salinity conditions is an important prerequisite for assessing more complicated ecological situations. As expected, Gammarus aequicauda shows a high resistance to abrupt changes in salinity. The present study shows that G. aequicauda can survive and grow in a wide range of salinities between 2 psu and 40 psu. These values are similar to those reported in other euryhaline peracarida species, such as the isopod Sphaeroma serratum Fabricius [37], the tanaidacea Tanais cavolini Milne-Edwards [38] and the amphipods Hyale crassicornis Haswell [39], Traskorchestia traskiana Stimpson [40], Orchesia gammarellus Pallas [41], Orchesia chilensis Milne-Edwards [42], Cyathura polita Stimpson [6, 43] and G. duebeni Lillegbørg [44].

The limits of tolerance depend upon the conditions of acclimation. When Gammarus aequicauda has been acclimated at low salinity (9 psu), survival at oligohaline conditions is greatly increased. Gradual acclimation over long intervals of time resulted in better survival in amphipods [29]. The present results contrast with those obtained by Prato et al. [24]. According to these authors, a high percentage of G. aequicauda acclimated to 36 psu can survive.
at low salinities (0 psu and 3 psu) for 10 days without any prior gradual acclimation to lower salinities. We have not been able to repeat Prato et al. results with individuals (adults or juveniles) of the Ebro delta population, perhaps due to possible intraspecific differences. Intraspecific geographical variations have been observed in other gammarid species, such as physiological and geographical differences between Ireland and Britain populations of *G. duebeni* [44]. It is possible that populations of *G. aequicauda* from Mar Piccolo (Italy) have a higher capability for compensatory adjustments to rapid salinity changes than *G. aequicauda* from Ebro delta.

Kevrekidis et al. [14] suggested that the growth and reproductive biology of *G. aequicauda* are not markedly affected by low salinities. According to the present results, *G. aequicauda* could well tolerate the salinities that were reported in the study area (4–37 psu) [13]. Within this range of salinities, this species can survive, reproduce, and grow in the laboratory. These results are consistent with those of Delgado et al. [13] who did not find a correlation between monthly *G. aequicauda* abundance and salinity values. It is known that parameters such as temperature and infection with parasites can change the range of salinity tolerance in amphipods [29, 39, 45]. Therefore, other factors such as temperature, oxygen concentration, predation, and pollution should also be considered in future studies.

The daily increases in BL obtained in this study (80–143 µm d⁻¹; estimated from logBL = 1.211logCL + 0.2197) are high compared with the values previously recorded by Delgado et al. [13] from their polymodal frequency distribution analyses (21–99 µm Bd⁻¹). This discrepancy is likely due to sampling biases that interfere with the
frequency distribution analyses. Although the growth rate values obtained in this study agree with those reported by Kevrekidis et al. [14] (50–150 µm d\(^{-1}\)) and Greze [18] (80–150 µm d\(^{-1}\)), these values are comparable to those reported in other amphipods. For instance, the growth rates of *Hyale crassicornis* were between 44 µm d\(^{-1}\) and 114 µm d\(^{-1}\) [39]. The lower growth rate values for *G. aequicauda* were obtained at extreme salinities (2 psu and 40 psu). It is likely that there is a higher energy requirement for osmoregulation under osmotic stress, which reduces the energy available for growth. D. H. Steele and V. J. Steele [3] observed a reduced growth rate to maturity in *Gammarus lawrenceanus* with decreasing salinity (from 15–20 psu to 2.5 psu). Normant et al. [46] reported similar values for *G. oceanicus*.

Under laboratory conditions, *G. aequicauda* reaches sexual maturity at 42 d when females reach 6–7 mm in BL. Delgado et al. [13] reported that the minimum size of an ovigerous female was 5 mm. These results are similar to those obtained for other *Gammarus* species. For example, *G. locusta* reared in the laboratory at 20°C and 33 psu salinity becomes sexually mature at 35 d, whereas at 15°C and 20–33 psu salinities, age at maturity was estimated to be 49 d [47].

In conclusion, *Gammarus aequicauda* can adapt to a wide range of salinity conditions, allowing juveniles to grow in many habitats under natural conditions. Growth of *G. aequicauda* juveniles is optimal at 4–34 psu salinities and 17°C–18°C. Thus, the *G. aequicauda* life history was not markedly affected by salinity changes if changes are not extremely sharp.

Acknowledgment

Financial support to G. Guerao (postdoctoral fellowship; INIA) was provided by the Ministry of Science and Research.

References


Research Article

Reproductive Traits of Some Amphipods (Crustacea: Peracarida) in Different Habitats of Iran and Southern Caspian Sea

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Received 20 December 2010; Revised 16 February 2011; Accepted 12 April 2011

Academic Editor: Chris Lloyd Mills

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Reproductive traits of seven amphipods from northern parts of Iran at 11 localities were studied in order to find feasible species for usage in aquaculture industries. The results revealed that the breeding season for *Gammarus lacustris* and *G. paricrenatus* from high latitudes was limited to a few months from May to June. Breeding activity of *G. komareki* from some springs was observed throughout the year, while *Obesogammarus acuminatus* and *G. aequicauda* from southern wetlands of the Caspian Sea and *Pontogammarus maeoticus* and *P. borceae* from southern Caspian sea shore showed various patterns. The mean egg number of *O. acuminatus* and *G. aequicauda* species was the highest with 49.8 and 37.7, respectively, while this value for *G. komareki* and *P. maeoticus* was the lowest with an average of 8.8 eggs. Reproductive strategy was found to be related to habitat characteristics such as chemical factors, substrate status, and the epifaunal living.

1. Introduction

Amphipods are often selected as test organisms in different studies because of their widespread distribution, sensitivity to disturbance, and culture suitability [1]. Introduction of macroinvertebrates such as crustacean to fish culture ponds has been used to improve fish diet, and many amphipods have been used [2]; others can be found in the website (http://www.elacuarista.com/). Amphipod adaptation and continuous reproduction in the new environment are the main factors for success.

A wide variation in reproductive parameters is found within amphipods, correlated with a wide variety of microhabitat conditions. A wide variation in reproductive parameters is found within amphipods corresponding to diversity of microhabitats conditions. In this regard, many comparisons of reproductive traits were explained by Nelson [3] for brackish water versus freshwater and marine, single-brooded versus multiple-brooded, and infaunal versus epifaunal species. However, an extensive literature has been published on the biology and life cycles of different amphipods; the studies of Nelson [3], Kolding and Fenchel [4], and Sainte-Marie [5] are informative about reproductive patterns. According to Sainte-Marie [5] life histories of gammarideans fall into eight categories. Most of them are an iteroparous annual type; the high reproductive potentials were described for semiannual populations in low latitude habitats while low reproductive potentials were more frequent in perennial gammarideans at high latitudes.

During a project [6] to find suitable species of amphipods for aquaculture industries, the reproductive traits of Amphipoda were examined in different habitats from the northern part of Iran. The selected species were *Gammarus lacustris* G. O. Sars, 1863, *G. paricrenatus* Stock et al. 1998, *G. komareki* Schäferna, 1992, *G. aequicauda* Martynov, 1931, *Obesogammarus acuminatus* Stock et al. 1998, *Pontogammarus maeoticus* (Sowinsky, 1894), and *P. borceae* (Carasus, 1943) from Iranian inland waters and southwest coast of the Caspian sea. All species had been confirmed previously by Stock et al. [7]. It was important to investigate their life cycle and breeding season in their respective habitats as reproductive traits vary with local water environment parameters.
2. Study Area and Methods

Different features of reproduction were studied in seven Amphipoda species from 11 habitats in the northern part of Iran (Table 1, Figure 1) mostly from the Caspian drainage basin. On the Iranian coast of the Caspian Sea *Pontogammarus maeoticus* is the most abundant and widely distributed [8], and *P. borceae* is much less abundant [9], both of which are considered in this study. Generally *P. maeoticus* inhabits the sublittoral region and is known from the Pontic and Azov Sea basins [10] while *P. borceae* has already been identified from the Pontic area with rheophilous characteristics [11]. *Gammarus komareki* from small streams and springs is the most common freshwater gammarid [7, 12] in the northern part of Iran. It is also distributed in Bulgaria, the northern part of Greece and Turkey, and around the Black sea [12]. At the south east of the Caspian sea in Gorgan Bay the common Mediterranean species *G. aequicauda* introduced to Caspian water before 1994 [13] is also considered.

The specimens were collected monthly from April 2001 to March 2002, though a few samples were missed in Neur Lake and Gorigol Wetland due to heavy snow and ice coverage of the Lake. Stock et al. [7] reported *G. lacustris* in Neur Lake and many other sites, previously recorded from Iran [14]. This species is widely distributed throughout the world and prefers mountain and glacier Lakes as habitats [12, 15]. In the Gorigol Wetland, *Gammarus paricrenatus* has been described as a new species [7]. On the other hand, no specimens of *Obesogammarus acuminatus* were found in Anzali Wetland for a few months.

The sampling was conducted by an Ekman dredge for muddy substrate or by a handle sieve (0.125 mm screen) for the aquatic vegetation and sandy substrates. The animals were washed from debris and preserved in 75% alcohol. In the laboratory, the length of the animal was measured to the nearest 1 mm from the anterior head margin to the posterior margin of the telson with a stereomicroscope fitted with a graticule. Animals were classified into five groups. Sex determination was according to the presence of oostegites. Breeding periods, clutch sizes, and ovigerous females percentage determined the breeding activity during the year. Juveniles in the brood pouch were assessed separately. The sex ratio was calculated for adults (longer than eight mm), and the mean length of adults was compared for each sex using a *t*-test. For multiple comparison of means ANOVA was used for some data analysis. Some environment characteristics such as hydrochemical parameters were measured by standard methods [16] to show the habitats variation.

3. Results

Biometric results showed a significant difference between the mean length of males and females where in most species males were longer than females, although *Pontogammarus maeoticus* was larger than males (Table 2). The sex ratios were highly variable for all species during the year while in the adult stage the male dominated. In *P. borceae* the sex ratio was 1 : 1.1, 1 : 1.2 for *G. paricrenatus*, *G. komareki*, *G. aequicauda*, *O. acuminatus* and 1 : 2.2 for *P. maeoticus* (Table 2).

The length-frequency histogram (Figure 2) of *Gammarus lacustris* shows that newly hatched (<4 mm long) individuals dominate with 43% and 23% of the population in June and July (Figure 3), respectively. They grow during the following months to juveniles with lengths of 4–8 mm, and their frequency in the population was 38% to 65% during August and October, respectively. The larger specimens (>12 mm) constituted the highest abundance in April and May with 84% and 37%, respectively. The newly hatched (<4 mm long) individuals of *G. paricrenatus* (Figure 3) dominated in May–July and comprised 30% to 49% of the population. They grow during the following months as the juveniles (4–8 mm long) make up 65% of the population during August. The larger specimens (>12 mm) show the highest frequency of 72% in March (Figure 2). The maximum mean length was observed in May and the minimum in June (Figure 4).

Studies on *G. aequicauda* Martynov, 1931 (Figure 2) revealed that newly hatched (<4 mm long) individuals present all year round while the frequency of juvenile (4–8 mm long) in the population was more than 50% (Figure 3). The larger specimens (>12 mm) constituted only 20 to 30% of the population in most periods, and the largest specimen was 19 mm in length.

Length-frequency histograms of *O. acuminatus* revealed that newly hatched and juvenile (<8 mm long) individuals were present in all sampling periods (Figure 3) when
the larger specimens (>12 mm) had the least frequency. The maximum and minimum mean length was observed in May and June, respectively, (Figure 4). The newly hatched and juvenile (<6 mm long) individuals of P. macoicus (Figure 2) dominated in most of the months (from 24 to 76% of the population). The maximum mean length was observed in May and the minimum in October (Figure 4). Individuals of P. borceae less than 6 mm long (Figure 2) were recorded throughout the year while the large specimens (>9 mm) were dominant in spring and winter (from 16 to 38% of the population). The maximum mean length was recorded in May and the minimum in January (Figure 4).

There was a significant difference in mean length during the year for all species (Figure 4), and according to Figure 3 the peak of abundance of newly hatched and juvenile length classes was in May to July for G. lacustris and G. paricrenatus while other species showed various patterns during the year. Furthermore the greatest percentage of ovigerous females of G. lacustris was observed in May 2001 while it was in April for G. paricrenatus (Figure 4).
<table>
<thead>
<tr>
<th>Species</th>
<th>Length (mm)</th>
<th>Egg (num/ind.)</th>
<th>Ovigerous female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. paricrenatus</em></td>
<td>F = 75.33, df = 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. lacustris</em></td>
<td>F = 154.07, df = 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. komareki</em></td>
<td>F = 15.75, df = 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. borceae</em></td>
<td>F = 26.97, df = 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. maeoticus</em></td>
<td>F = 23.19, df = 11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4: Continued.
Figure 4: Mean length (mm), fecundity (egg/ind.), and frequency of ovigerous female for each species during April 2001 to March 2002.

Table 1: Sampling localities for amphipods of the present study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sampling site</th>
<th>Position</th>
<th>Altitude</th>
<th>Studied specimens number</th>
<th>Sampling method*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gammarus lacustris</em> G.O. Sars, 1863</td>
<td>Neur Lake (3 points)</td>
<td>38° 00’ N, 48° 35’ E</td>
<td>2500 m</td>
<td>1158</td>
<td>From substrate by E.d</td>
</tr>
<tr>
<td><em>Gammarus paricrenatus</em> Stock et al. 1998</td>
<td>Gorigol Wetland (3 points)</td>
<td>37° 54’ N, 46° 42’ E</td>
<td>1330 m</td>
<td>1465</td>
<td>From aquatic plant by H.s</td>
</tr>
<tr>
<td><em>Gammarus komareki</em> Schaferna, 1992</td>
<td>Springs; Hiran</td>
<td>38° 24’ N, 48° 44’ E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Navrud</td>
<td>37° 42’ N, 48° 51’ E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sefidab</td>
<td>37° 00’ N, 50° 18’ E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kojor</td>
<td>36° 23’ N, 51° 43’ E</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gammarus aequicauda</em> Martynov, 1931</td>
<td>Gorgan Bay (4 points)</td>
<td>36° 50’ N, 53° 45’ E</td>
<td>−10 m</td>
<td>1215</td>
<td>From edge aquatic plant by H.s</td>
</tr>
<tr>
<td><em>Obesogammarus acuminatus</em> Stock et al. 1998</td>
<td>Anzali Wetland (4 points)</td>
<td>37° 29’ N, 49° 21’ E</td>
<td>−5 m</td>
<td>518</td>
<td>From aquatic plant by H.s</td>
</tr>
<tr>
<td><em>Pontogammarus maeoticus</em> (Sowinsky, 1894)</td>
<td>Caspian sea shore; Astara, Sefid Rud estuary</td>
<td>38° 26’ N, 48° 53’ E 37° 27’ N, 49° 55’ E</td>
<td>2014</td>
<td></td>
<td>From substrate by E.d</td>
</tr>
<tr>
<td><em>Pontogammarus boreae</em> (Carasu, 1943)</td>
<td>Kelachi</td>
<td>37° 05’ N, 50° 26’ E</td>
<td>−10 m</td>
<td>907</td>
<td></td>
</tr>
</tbody>
</table>

*E.d.: Ekman dredge and H.s.: handle sieve.*
Figure 5: Linear regression of egg number in brood pouch with female size for each species.
The numbers are examined presented in the parenthesis. had a few eggs per female with 8. maeoticus. The highest fecundity with mean egg numbers of 37 (Figure 4). was also distinguished from August to November, increased effectively (Figure 2). The least percentage of ovigerous females all months of the year but the maximum and minimum during April 2001 to March 2002.

Table 2: Mean length of male and female specimens for each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean length ± SEa</th>
<th>t-valueb</th>
<th>Sex ratio (F/M)</th>
<th>Min. and max. length of ovigerous female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gammarus lacustris</td>
<td>F = 12.42 ± 0.12</td>
<td>2.26*</td>
<td>1 : 1.5</td>
<td>8–16.7</td>
</tr>
<tr>
<td>G.O. Sars, 1863</td>
<td>M = 12.87 ± 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammarus paricrenatus</td>
<td>F = 11.01 ± 0.13</td>
<td>5.43*</td>
<td>1 : 1.2</td>
<td>10–14.5</td>
</tr>
<tr>
<td>Stock et al. 1998</td>
<td>M = 12.04 ± 0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammarus komareki</td>
<td>F = 7.65 ± 0.04</td>
<td>16.64*</td>
<td>1 : 1.2</td>
<td>5.5–12</td>
</tr>
<tr>
<td>Schaferna, 1992</td>
<td>M = 8.71 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammarus aequicauda</td>
<td>F = 9.94 ± 0.11</td>
<td>6.74*</td>
<td>1 : 1.2</td>
<td>5.4–14</td>
</tr>
<tr>
<td>Martynov, 1931</td>
<td>M = 10.94 ± 0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesogammarus acuminatus</td>
<td>F = 11.03 ± 0.21</td>
<td>1.17</td>
<td>1 : 1.2</td>
<td>7.2–16.7</td>
</tr>
<tr>
<td>Stock et al. 1998</td>
<td>M = 11.43 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pontogammarus maeoticus</td>
<td>F = 8.39 ± 0.09</td>
<td>1.47</td>
<td>1 : 1.5</td>
<td>8.5–15</td>
</tr>
<tr>
<td>(Sowinsky, 1894)</td>
<td>M = 8.60 ± 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pontogammarus borcae</td>
<td>F = 8.62 ± 0.10</td>
<td>0.23</td>
<td>1 : 1</td>
<td>5–13.3</td>
</tr>
<tr>
<td>(Caraus, 1943)</td>
<td>M = 8.60 ± 0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The measurements did not include newly hatched and juvenile individuals.

The numbers are examined presented in the parenthesis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Correlation value (Sig. level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. paricrenatus</td>
<td>−0.22 (0.78)</td>
</tr>
<tr>
<td>G. lacustris</td>
<td>0.97 (0.17)</td>
</tr>
<tr>
<td>G. komareki</td>
<td>0.15 (0.65)</td>
</tr>
<tr>
<td>P. borcae</td>
<td>−0.02 (0.96)</td>
</tr>
<tr>
<td>P. maeoticus</td>
<td>0.65 (0.04)*</td>
</tr>
<tr>
<td>G. aequicauda</td>
<td>−0.46 (0.13)</td>
</tr>
<tr>
<td>O. acuminatus</td>
<td>−0.41 (0.42)</td>
</tr>
</tbody>
</table>

*Correlation is significant at the .05 level (2-tailed). females and brood size of P. maeoticus were observed in mid-spring (Figure 4). The maximum egg number per female was observed in G. aequicauda (225 eggs), and its ovigerous female percentages were larger in spring than in winter while the mean brood size was vice versa (Figure 4). The mean number of eggs of G. lacustris and P. borcae was 10.2 ± 5.2 and 14.9 ± 13.1, respectively.

According to Figure 5, body length was linearly significantly correlated with number of eggs per female for species of O. acuminatus (r = 0.765, P < .01), G. aequicauda (r = 0.710, P < .01), P. maeoticus (r = 0.504, P < .01), P. borcae (r = 0.664, P < .01), and G. komareki (r = 0.404, P < .01). There was no significant correlation between egg number and individual length for G. lacustris (r = 0.122, P > .1) and G. paricrenatus (r = 0.23, P > .1). Only in P. maeoticus did the mean number of eggs correlate to the percentage of ovigerous females (Table 3).

Results of habitat characteristics show that Neur and Gorgan freshwater Lakes are located at high altitude (more than 1330 metres above sea level) and their salinities are 0.3 and 0.7 ppt., respectively, (Table 4); the water temperature...
in Gorigol varies between 0 and 30°C which is slightly higher than that in Neur (0–23.6°C). The oxygen level is also higher in Gorigol than that in Neur (Figure 6). The pH in Gorigol is more alkaline than Neur (Table 4). The coastal habitants (Astara, Sefid Rud estuary, and Kelachi), Anzali Wetland and Gorgan Bay are −10 to −5 meters below sea level. The Gorgan Bay, is the most saline (15.8 ± 5.7 ppt) followed by coastal area (7.1 ± 4.15 ppt) and Anzali Wetland (3.2 ± 2.8 ppt). The temperature (Figure 6) in Anzali Wetland 20.2 ± 7.6°C was higher than that in the coastal area (18.2 ± 7.8°C); the pH values in the coastal area and Gorgan Bay were relatively similar (about 8.3) but in Anzali Wetland the pH was barely neutral (7.7) (Table 4).

The oxygen level in Anzali Wetland was lower than that in coastal area and Gorgan Bay (Figure 6), and dissolved inorganic nitrogen (DIN) in Gorigol and Anzali Wetlands total nitrogen (TN) in Neur Lake and Gorgan Bay had the highest concentrations. Compared to other sites (Table 4), Neur lake showed the most phosphorous components consist of total phosphorus (TP) and dissolved inorganic phosphorus (DIC). The springs Hirran, Navrud, and Kojar were mainly located in mountainous areas well above sea level; their salinities are low so they are almost freshwater habitats. The temperature is relatively low (12.8 ± 5.4°C) with respect to other habitats. Oxygen concentration is high (10.9 mg/L) and their pH is almost neutral (7.74).

### Table 4: Some abiotic characteristics of the habitats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Neur Lake</th>
<th>Gorigol Wetland</th>
<th>Springs</th>
<th>Gorgan Bay</th>
<th>Anzali Wetland</th>
<th>Caspian sea shore</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIN (mg/L)*</td>
<td>0.36</td>
<td>0.69</td>
<td>0.57</td>
<td>0.19</td>
<td>0.67</td>
<td>0.3</td>
</tr>
<tr>
<td>TN (mg/L)*</td>
<td>1.87</td>
<td>1.42</td>
<td>0.84</td>
<td>1.52</td>
<td>1.34</td>
<td>0.71</td>
</tr>
<tr>
<td>DIP (mg/L)*</td>
<td>0.16</td>
<td>0.05</td>
<td>0.07</td>
<td>0.04</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>TP (mg/L)*</td>
<td>0.38</td>
<td>0.10</td>
<td>0.11</td>
<td>0.14</td>
<td>0.2</td>
<td>0.11</td>
</tr>
<tr>
<td>Ca (mg/L)*</td>
<td>50</td>
<td>22</td>
<td>57</td>
<td>331</td>
<td>99</td>
<td>216</td>
</tr>
<tr>
<td>Mg (mg/L)*</td>
<td>15</td>
<td>21</td>
<td>11</td>
<td>661</td>
<td>143</td>
<td>397</td>
</tr>
<tr>
<td>Total Hardness (mg/L)*</td>
<td>131</td>
<td>141</td>
<td>195</td>
<td>3620</td>
<td>774</td>
<td>2293</td>
</tr>
<tr>
<td>Range of pH</td>
<td>8.4–8.9</td>
<td>9.1–10.9</td>
<td>5.8–10.4</td>
<td>7.6–8.7</td>
<td>6.8–8.3</td>
<td>7.8–8.5</td>
</tr>
<tr>
<td>Range of Salinity (ppt)</td>
<td>0.2–0.3</td>
<td>0.4–1.4</td>
<td>0.2–0.4</td>
<td>5.6–22.6</td>
<td>0.6–7.5</td>
<td>3.2–10.7</td>
</tr>
</tbody>
</table>

* From a single sampling during October 2001.

4. Discussion

The sex ratio and domination of males in most adult populations in this study probably resulted from better survival and faster growth rate. This is also obvious in their size difference (Table 2). In most gammarid amphipods, sexual dimorphism is observed and this was previously proved in G. chevreuxi [1]. However biotic and abiotic environmental factors have an effect on sex ratio as the study by Steele and Steele [17] on Gammarus dubeni showed that low temperature results in males, high temperatures produce females, and during the rest of year the sex ratio is usually equal.

In this study Gammarus komareki and G. aequicauda demonstrated continuous breeding throughout the year, as indicated by the number of juveniles present in the smaller size classes and the ovigerous females, observed in many species [18]. The population of G. komareki showed an iteroparous annual life cycle where juvenile recruitment peaked in winter and mid-spring. This characteristic dominated in G. aequicauda populations during spring. The reproductive pattern of P. maeoticus showed two seasonal peaks in January and June-July, which was previously observed [8]. This was also recorded for the north Caspian Sea population of P. maeoticus [2]. Three peaks of ovigerous females of two sandhoppers which are indicative of more than one generation in a year were observed by Charfi-Cheikhrouha et al. [19], and they suggested that this continuous breeding activity occurs in the southern geographic distribution of populations. More generations per year of Calliopius laeviusculus have been observed in the southern populations, rather than a single generation in the northern ones [20].

Individuals of G. locusta produce two generations each year, in winter and summer [21], while Jazzdewski [22] reported production of three generations in spring, summer, and autumn each year for this species. According to Steele and Steele [20], temperature shows marked influence on life and reproduction of individuals. The higher temperature provides favourable condition for faster growth and development resulted in occurrence of multiple generations annually. This was confirmed with G. komareki, P. maeoticus, and G. aequicauda in this study. Moreover the low temperature variation in springs and small streams (Figure 6) caused the continuous reproduction in G. komareki. Steele and Steele [17] also showed that high summer temperatures, particularly promote rapid growth of G. dubeni and allow a second generation to breed. Showing continuous breeding same as other species [1, 4], many peaks of reproductive activity in this species support the idea of multiple breeding during their life.

Conversely, both G. lacustris and G. paricrenatus have a simple annual breeding cycle, with a reproductive peak in May to July (Figure 2). The water temperature varies widely between the habitats (Figure 6) where the high latitudes and low temperatures of winter result in slow growth and development and the production of a few or one generation per year. Similarly, in the life cycle of Synurella ambulans from central ponds of Poland which were covered with ice during winter, hatching occurs in summer, individuals
in burrow mostly immobile, and feeding on interstitial microflora and microfauna resulted in reduction in body size in infaunal benthic animals [3]. In this study, the pattern of increasing brood size with body size (Figure 5) is probably a general trend in malacostracan Crustacean including Amphipoda as stated by Nelson [3]. Several studies on life cycle of freshwater gammarids revealed a significant correlation between female length and the number of eggs [23, 29]; our finding also demonstrate similar relationships between female size and egg numbers for G. aequicauda, O. acuminatus, P. borceae, G. komareki, and P. maeoticus. However, in G. lacustris and G. paricrenatus, the number of eggs did not significantly increase with the length of the female. Synurella ambulans also exhibited a similar pattern [23].

According to these field observations and the supplementary laboratory experiments [6] it was concluded that four species, P. maeoticus, P. borceae, O. acuminatus, and G. aequicauda, seem to be good candidates for potential use in warm water fish farms. However, a subsequent study [30] produced negative results as Amphipoda replacement in fish culture ponds was not satisfactory with most animals dead within a few days. The hydrochemical parameters including poor oxygen concentration and very high nutrient levels were the factors responsible for mortality of specimens. In conclusion, future work is needed to determine whether P. maeoticus can adapt to fully freshwater environments.

Acknowledgments

The authors are grateful to H. Khodaparast Sharifi who provided useful comments on an earlier draft. The study was supported through project no. 79-0710340000-04 from the Iranian Fisheries Research Institute, and the authors would like to thank all persons responsible especially A. Danesh the previous Vice President of the Inland Water Aquaculture Research Centre.

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