International Journal of Zoology

Gammarus: Important Taxon in Freshwater and Marine Changing Environments

Guest Editors: Almut Gerhardt, Michelle Bloor, and Chris Lloyd Mills

Gammarus: Important Taxon in Freshwater and Marine Changing Environments

International Journal of Zoology

Gammarus: Important Taxon in Freshwater and Marine Changing Environments

Guest Editors: Almut Gerhardt, Michelle Bloor, and Chris Lloyd Mills

Editorial Board

Contents

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 524276, 2 pages
doi:10.1155/2011/524276

Editorial

Gammarus: Important Taxon in Freshwater and Marine Changing Environments

Almut Gerhardt,[1] Michelle Bloor,[2] and Chris Lloyd Mills[3]

[1] *LimCo International GmbH, Technologiezentrum Konstanz, Blarerstrβe 56, 78462 Konstanz, Germany*
[2] *School of Earth and Environmental Science, University of Portsmouth, Burnaby Building, Burnaby Road, Portsmouth PO1 3QL, UK*
[3] *School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK*

Correspondence should be addressed to Almut Gerhardt, almutg@web.de

Received 19 June 2011; Accepted 19 June 2011

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.

Almut Gerhardt
Michelle Bloor
Chris Lloyd Mills

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 295026, 6 pages
doi:10.1155/2011/295026

Review Article

Gammarus-Microbial Interactions: A Review

Daniel Nelson

Aquatic Biology Program, Department of Biological Sciences, The University of Alabama, 1106 Bevill Building, 201 Seventh Avenue, P.O. Box 870206, Tuscaloosa, AL 35487, USA

Correspondence should be addressed to Daniel Nelson, dnelson12@crimson.ua.edu

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

Academic Editor: Almut Gerhardt

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. troglophilus* consumed conditioned oak if they were the only leaves presented to it [31]. However, if presented with conditioned oak and elm leaves simultaneously, *G. troglophilus* 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

[1] C. Macneil, J. T. A. Dick, and R. W. Elwood, "The trophic ecology of freshwater *Gammarus* spp. (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept," *Biological Reviews of the Cambridge Philosophical Society*, vol. 72, no. 3, pp. 349–364, 1997.

[2] G. W. Minshall, "Role of allochthonous detritus in the trophic structure of a wodland springbrook community," *Ecology*, vol. 48, pp. 139–149, 1967.

[3] D. Nelson, *Food web structure of cave streams in southwestern Illinois and the survival and growth of the stygophilic Gammarus troglophilus (Crustacea: Amphipoda) under laboratory conditions*, M.S. thesis, University of Idaho, Moscow, Idaho, USA, 2010.

[4] J. W. Moore, "The role of algae in the diet of *Asellus aquaticus* L. and *Gammrus pulex* L," *Journal of Animal Ecology*, vol. 44, pp. 719–730, 1975.

[5] H. Orav-Kotta, J. Kotta, K. Herkül, I. Kotta, and T. Paalme, "Seasonal variability in the grazing potential of the invasive amphipod *Gammarus tigrinus* and the native amphipod *Gammarus salinus* (Amphipoda: Crustacea) in the northern Baltic Sea," *Biological Invasions*, vol. 11, no. 3, pp. 597–608, 2009.

[6] D. C. Culver and D. W. Fong, "Species interactions in cave stream communities: experimental results and microdistribution effects," *American Midland Naturalist*, vol. 126, pp. 364–379, 1991.

[7] F. M. Wilhelm and D. W. Schindler, "Effects of *Gammarus lacustris* (Crustacea: Amphipoda) on plankton community structure in an alpine lake," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 56, no. 8, pp. 1401–1408, 1999.

[8] J. T. A. Dick, I. Montgomery, and R. W. Elwood, "Replacement of the indigenous amphipod Gammarus duebeni celticus by the introduced G. pulex: differential cannibalism and mutual predation," *Journal of Animal Ecology*, vol. 62, no. 1, pp. 79–88, 1993.

[9] J. T. Dick, "The cannibalistic behaviour of two *Gammarus* species (Crustacea: Amphipoda)," *Journal of Zoology*, vol. 236, no. 4, pp. 697–706, 1995.

[10] K. W. Cummins, "Trophic relations of aquatic insects," *Annual Review of Entomology*, vol. 18, pp. 183–206, 1973.

[11] K. W. Cummins, "Structure and function of stream ecosystems," *Bioscience*, vol. 24, pp. 631–641, 1974.

[12] K. W. Cummins and M. J. Klug, "Feeding ecology of stream invertebrates," *Annual Review of Ecology and Systematics*, vol. 10, pp. 147–172, 1979.

[13] J. L. Meyer, "The microbial loop in flowing waters," *Microbial Ecology*, vol. 28, no. 2, pp. 195–199, 1994.

[14] R. O. Hall and J. L. Meyer, "The trophic significance of bacteria in a detritus-based stream food web," *Ecology*, vol. 79, no. 6, pp. 1995–2012, 1998.

[15] K. S. Simon and E. F. Benfield, "Leaf and wood breakdown in cave streams," *Journal of the North American Benthological Society*, vol. 20, no. 4, pp. 550–563, 2001.

[16] R. H. Boling, E. D. Goodman, J. O. Zimmer et al., "Toward a model of detritus processing in a woodland stream," *Ecology*, vol. 56, pp. 141–151, 1975.

[17] T. L. Arsuffi and K. Suberkropp, "Selective feeding by shredders on leaf-colonizing stream fungi: comparison of macroinvertebrate taxa," *Oecologia*, vol. 79, no. 1, pp. 30–37, 1989.

[18] K. S. Simon, E. F. Benfield, and S. A. Macko, "Food web structure and the role of epilithic biofilms in cave streams," *Ecology*, vol. 84, no. 9, pp. 2395–2406, 2003.

[19] F. Bärlocher and B. Kendrick, "Fungi and food preferences of *Gammarus pseudolimnaeus*," *Archiv für Hydrobiologie*, vol. 72, pp. 501–516, 1973.

[20] M. Kostalos and R. L. Seymour, "Role of microbially enriched detritus in the nutrition of *Gammarus minus* (Amphipoda)," *Oikos*, vol. 27, pp. 512–516, 1976.

[21] Q. Rong, K. R. Sridhar, and F. Bärlocher, "Food selection in three leaf-shredding stream invertebrates," *Hydrobiologia*, vol. 316, no. 3, pp. 173–181, 1995.

[22] C. Assmann and E. V. Elert, "The impact of fungal extracts on leaf litter on the food preference of *Gammarus roeselii*," *International Review of Hydrobiology*, vol. 94, no. 4, pp. 484–496, 2009.

[23] M. Pöckl, "Laboratory studies on growth, feeding, moulting and mortality in the freshwater amphipods *Gammarus fossarum* and *G. roeseli*," *Archiv für Hydrobiologie*, vol. 134, no. 2, pp. 223–253, 1995.

[24] M. A. S. Graça, L. Maltby, and P. Calow, "Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus*. II. Effects on growth, reproduction and physiology," *Oecologia*, vol. 96, no. 3, pp. 304–309, 1993.

[25] F. Bärlocher, "On trophic interactions between microorganisms and animals," *The American Naturalist*, vol. 114, pp. 147–148, 1979.

[26] P. Kemp, "Potential impact on bacteria of grazing by a macrofaunal deposit-feeder, and the fate of bacterial production," *Marine Ecology Progress Series*, vol. 36, pp. 151–161, 1987.

[27] S. J. Morrison and D. C. White, "Effects of grazing by estuarine gammaridean amphipods on the microbiota of allochthonous detritus," *Applied and Environmental Microbiology*, vol. 40, pp. 659–671, 1980.

[28] M. L. Pace and J. J. Cole, "Regulation of bacteria by resources and predation tested in whole-lake experiments," *Limnology and Oceanography*, vol. 41, no. 7, pp. 1448–1460, 1996.

[29] T. J. Cooney and K. S. Simon, "Influence of dissolved organic matter and invertebrates on the function of microbial films in groundwater," *Microbiology of Aquatic Systems*, vol. 58, pp. 599–610, 2009.

[30] J. Kinsey, T. J. Cooney, and K. S. Simon, "A comparison of the leaf shredding ability and influence on microbial films of surface and cave forms of *Gammarus minus* Say," *Hydrobiologia*, vol. 589, no. 1, pp. 199–205, 2007.

[31] F. Jenio, "The life cycle and ecology of *Gammarus troglophilus* Hubricht and Mackin," *Crustaceana Supplement*, vol. 6, pp. 204–215, 1980.

[32] T. M. Iversen, "Ingestion and growth in *Sericostoma personatum* (Trichoptera) in relation to the nitrogen content of ingested leaves," *Oikos*, vol. 25, no. 3, pp. 278–282, 1974.

[33] M. A. S. Graça, C. Cressa, M. O. Gessner, M. J. Feio, K. A. Callies, and C. Barrios, "Food quality, feeding preferences, survival and growth of shredders from temperate and tropical streams," *Freshwater Biology*, vol. 46, no. 7, pp. 947–957, 2001.

[34] F. J. Triska, *Seasonal distribution of aquatic hyphomycetes in relation to the disappearance of leaf litter from a woodland stream*, Ph.D. dissertation, University of Pittsburgh, Pittsburgh, Pa, USA, 1970.

[35] N. K. Kaushik and H. B. N. Hynes, "The fate of the dead leaves that fall into streams," *Archiv für Hydrobiologie*, vol. 68, pp. 465–515, 1971.

[36] J. S. Rounick and M. J. Winterbourn, "Leaf processing in two contrasting beech forest streams: effects of physical and biotic factors on litter breakdown," *Archiv für Hydrobiologie*, vol. 96, pp. 448–474, 1983.

[37] F. Bärlocher, "The role of fungi in the nutrition of stream invertebrates," *Botanical Journal of the Linnean Society*, vol. 91, pp. 83–94, 1985.

[38] K. Suberkropp, "Interactions with invertebrates," in *The Ecology of Aquatic Hyphomycetes*, F. Bärlocher, Ed., pp. 118–134, Springer, New York, NY, USA, 1992.

[39] M. A. S. Graça, "Patterns and processes in detritus-based stream systems," *Limnologica*, vol. 23, pp. 107–114, 1993.

[40] M. A. S. Graça, "The role of invertebrates on leaf litter decomposition in streams—a review," *International Review of Hydrobiology*, vol. 86, no. 4-5, pp. 383–393, 2001.

[41] N. Friberg and D. Jacobsen, "Feeding plasticity of two detritivore-shredders," *Freshwater Biology*, vol. 32, no. 1, pp. 133–142, 1994.

[42] M. A. S. Graça, L. Maltby, and P. Calow, "Importance of fungi in the diet of *Gammarus pulex* and *Asellus aquaticus* I: feeding strategies," *Oecologia*, vol. 93, no. 1, pp. 139–144, 1993.

[43] W. H. Karasov and C. Martinez del Rio, *Physiological Ecology*, Princeton University Press, Princeton, NJ, USA, 2007.

[44] F. Bärlocher and B. Kendrick, "Assimilation efficiency of *Gammarus pseudolimnaeus* (Amphipoda) feeding on fungal mycelium or autumn-shed leaves," *Oikos*, vol. 26, pp. 55–59, 1975.

[45] R. J. Barsdate, R. T. Prentki, and T. Fenchel, "Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers," *Oikos*, vol. 25, no. 3, pp. 239–251, 1974.

[46] L. E. Barnese, R. L. Lowe, and R. D. Hunter, "Comparative grazing efficiencies of pulmonate and prosobranch snails," *Journal of the North American Benthological Society*, vol. 9, pp. 35–44, 1990.

[47] G. J. C. Underwood and J. D. Thomas, "Grazing interactions between pulmonate snails and epiphytic algae and bacteria," *Freshwater Biology*, vol. 23, no. 3, pp. 505–522, 1990.

[48] N. V. C. Polunin, "The decomposition of emergent macrophytes in fresh water," in *Advances in Ecological Research, Volume 14*, A. Macfadyen and E. D. Ford, Eds., pp. 115–166, Academic Press, New York, NY, USA, 1984.

[49] J. R. Lawrence, B. Scharf, G. Packroff, and T. R. Neu, "Microscale evaluation of the effects of grazing by invertebrates with contrasting feeding modes on river biofilm architecture and composition," *Microbial Ecology*, vol. 44, no. 3, pp. 199–207, 2002.

[50] P. J. Mulholland, A. D. Steinman, E. R. Marzolf, D. R. Hart, and D. L. DeAngelis, "Effect of periphyton biomass on hydraulic characteristics and nutrient cycling in streams," *Oecologia*, vol. 98, no. 1, pp. 40–47, 1994.

[51] T. J. Battin, L. A. Kaplan, J. D. Newbold, and C. M. E. Hansen, "Contributions of microbial biofilms to ecosystem processes in stream mesocosms," *Nature*, vol. 426, no. 6965, pp. 439–442, 2003.

[52] R. W. Sterner, "Modelling interactions of food quality and quantity in homeostatic consumers," *Freshwater Biology*, vol. 38, no. 3, pp. 473–481, 1997.

[53] R. W. Sterner and J. J. Elser, *Ecological Stoichiometery: The Biology of Elements from Molecules to the Biosphere*, Princeton University Press, Princeton, NJ, USA, 2002.

[54] M. T. Brett and D. C. Müller-Navarra, "The role of highly unsaturated fatty acids in aquatic foodweb processes," *Freshwater Biology*, vol. 38, no. 3, pp. 483–499, 1997.

[55] M. Torres-Ruiz, J. D. Wehr, and A. A. Perrone, "Trophic relations in a stream food web: importance of fatty acids for macroinvertebrate consumers," *Journal of the North American Benthological Society*, vol. 26, no. 3, pp. 509–522, 2007.

[56] W. F. Cross, J. P. Benstead, P. C. Frost, and S. A. Thomas, "Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives," *Freshwater Biology*, vol. 50, no. 11, pp. 1895–1912, 2005.

[57] P. C. Frost, J. P. Benstead, W. F. Cross et al., "Threshold elemental ratios of carbon and phosphorus in aquatic consumers," *Ecology Letters*, vol. 9, no. 7, pp. 774–779, 2006.

[58] A. Liess and H. Hillebrand, "Stoichiometric variation in C:N, C:P, and N:P ratios of littoral benthic invertebrates," *Journal of the North American Benthological Society*, vol. 24, no. 2, pp. 256–269, 2005.

[59] M. Torres-Ruiz, J. D. Wehr, and A. A. Perrone, "Are net-spinning caddisflies what they eat? An investigation using controlled diets and fatty acids," *Journal of the North American Benthological Society*, vol. 29, no. 3, pp. 803–813, 2010.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 294394, 5 pages
doi:10.1155/2011/294394

Research Article

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

M. C. Bloor

School of Earth and Environmental Science, University of Portsmouth, Burnaby Building, Burnaby Road, Portsmouth, PO1 3QL, UK

Correspondence should be addressed to M. C. Bloor, michelle.bloor@port.ac.uk

Received 29 March 2011; Accepted 1 June 2011

Academic Editor: Chris Lloyd Mills

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 F^1, F^2, F^3 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.

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]).

Stock solution	g L^{-1}
$CaCl_2 \cdot H_2O$	58.80
$MgSO_4 \cdot 7H_2O$	24.65
$NaHCO_3$	12.95
KCl	1.15

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, $P < 0.001$)).

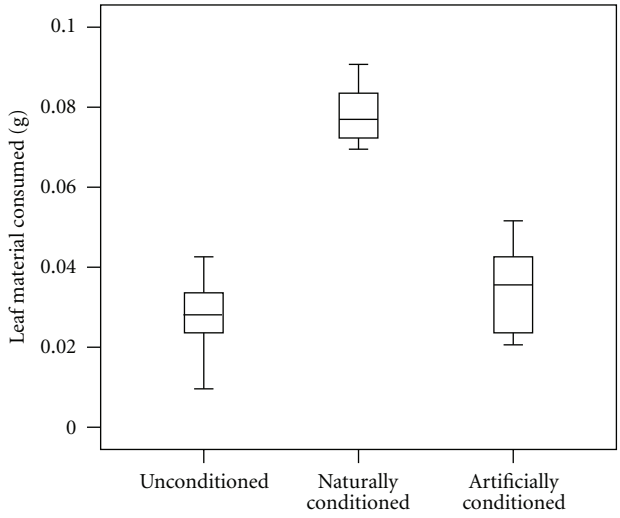


Figure 1: Box and whisker plot of leaf material consumed for each treatment (g) by *G. pulex*. The figure illustrates that during 24 hour feeding assays, *G. pulex* consumed a greater proportion of naturally conditioned leaf material, compared to artificially conditioned and unconditioned ($n = 10$ replicated tests for each treatment).

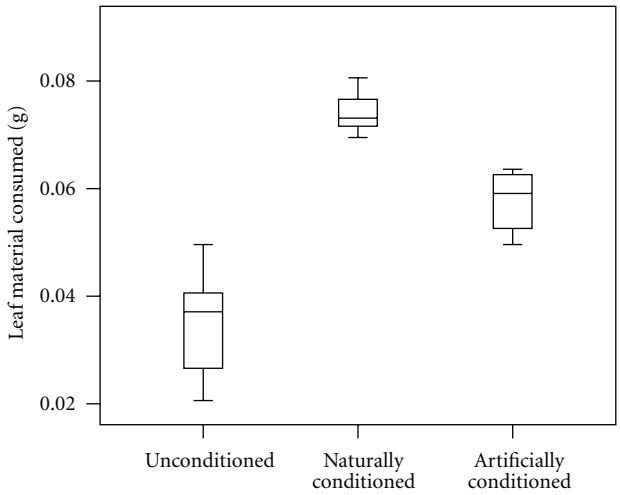


Figure 2: Box and whisker plot of leaf material consumed for each treatment (g) by *A. aquaticus*. The figure illustrates that during 24-hour feeding assays, *A. aquaticus* consumed a greater proportion of naturally conditioned leaf material, compared to the artificially conditioned and unconditioned ($n = 10$ replicated tests for each treatment).

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

[1] L. G. Willoughby and D. W. Sutcliffe, "Experiments on feeding and growth of the amphipod *Gammarus pulex* (L.) related to its distribution in the River Duddon," *Freshwater Biology*, vol. 6, no. 6, pp. 577–586, 1976.

[2] S. J. Blockwell, D. Pascoe, and E. J. Taylor, "Effects of lindane on the growth of the freshwater amphipod *Gammarus pulex* (L.)," *Chemosphere*, vol. 32, no. 9, pp. 1795–1803, 1996.

[3] M. C. Bloor, C. J. Banks, and V. Krivtsov, "Acute and sub-lethal toxicity tests to monitor the impact of leachate on an aquatic environment," *Environment International*, vol. 31, no. 2, pp. 269–273, 2005.

[4] M. C. Bloor, "Animal standardisation for mixed species ecotoxicological studies: establishing a laboratory breeding programme of *Gammarus pulex* and *Asellus aquaticus*," *Zoologica Baetica*, vol. 21, pp. 179–190, 2010.

[5] N. H. Anderson and J. R. Sedell, "Detritus processing by macroinvertebrates in stream ecosystems," *Annual Review of Entomology*, vol. 24, pp. 351–377, 1979.

[6] S. W. Gollady, J. R. Webster, and E. F. Benfield, "Factors affecting food utilization by a leaf shredding aquatic insect: leaf species and conditioning time," *Ecography*, vol. 6, no. 2, pp. 157–162, 1983.

[7] C. M. Bueler, "Feeding preference of *Pteronarcys pictetii* (Plecoptera: Insecta) from a small, acidic, woodland stream," *The Florida Entomologist*, vol. 67, no. 3, pp. 393–401, 1984.

[8] G. A. Rosenthal and D. H. Janzen, *Herbivores: Their Interaction with Secondary Plant Metabolites*, Academic Press, New York, NY, USA, 1979.

[9] M. A. S. Graca, L. Maltby, and P. Calow, "Comparative ecology of *Gammarus pulex* (L.) and *Asellus aquaticus* (L.) population dynamics and microdistribution," *Hydrobiologia*, vol. 281, no. 3, pp. 155–162, 1994.

[10] T. M. Iversen, "Ingestion and growth in *Sericostoma personatum* (Trichoptera) in relation to the nitrogen content of ingested leaves," *Oikos*, vol. 25, no. 3, pp. 278–282, 1974.

[11] L. M. Nilsson, "Energy budget of a laboratory population of *Gammarus pulex* (Amphipoda)," *Oikos*, vol. 25, no. 1, pp. 35–42, 1974.

[12] C. Naylor, L. Maltby, and P. Calow, "Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore," *Hydrobiologia*, vol. 188-189, no. 1, pp. 517–523, 1989.

[13] C. P. McCahon and D. Pascoe, "Culture techniques for three freshwater macroinvertebrate species and their use in toxicity tests," *Chemosphere*, vol. 17, no. 12, pp. 2471–2480, 1988.

[14] F. Barlocher and B. Kendrick, "Dynamics of the fungal population on leaves in a stream," *Journal of Ecology*, vol. 62, pp. 761–791, 1974.

[15] M. A. S. Graca, *Observations on the feeding biology of two stream-dwelling detritivores:* Gammarus pulex *(L.) and* Asellus aquaticus *(L.)*, Ph.D. thesis, University of Sheffield, South Yorkshire, UK, 1990.

[16] K. E. McGrath, E. T. H. M. Peeters, J. A. J. Beijer, and M. Scheffer, "Habitat-mediated cannibalism and microhabitat restriction in the stream invertebrate *Gammarus pulex*," *Hydrobiologia*, vol. 589, no. 1, pp. 155–164, 2007.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 574536, 7 pages
doi:10.1155/2011/574536

Research Article

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

Almut Gerhardt

LimCo International GmbH, Technologiezentrum Konstanz, Blarerstraße 56, 78462 Konstanz, Germany

Correspondence should be addressed to Almut Gerhardt, almutg@web.de

Received 4 February 2011; Accepted 9 April 2011

Academic Editor: Michelle Bloor

Gammarus spp. represent an important taxon in running water ecosystems concerning both structural and functional aspects. *Gammarus* spp. are also part of several macrozoobenthos indices for assessing biological water quality. However, in ecotoxicological water quality assessment, this taxon has been used much less than *Daphnia* spp. A new user-friendly and low-cost test protocol for *Gammarus* spp. has been developed, constituting the "ecotoxicological module "of an integrated multimetric triad-based concept for water quality assessment. The GamTox test is based on several test parameters: behavior (especially locomotion and feeding) depicts rapid and sensitive early warning indicators, survival displays an indicator of severe acute stress, and biochemical biomarkers, esp. AChE inhibition, is a sensitive marker of neurotoxic xenobiotic stress. GamTox can be performed both *in situ* and *ex situ*, based either on visual or automatical recording.

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 cannibalists. 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 exptrapolate 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*.

Substance (μg/L)	Daph-48	Gam-96	Daph/G	D/Gam	Daph-96 (2)	D/G-corr
Metals						
Cadmiumchloride	25	19		1,3	12,5	0,6
Copper	9	37	0,2		4,5	0,12
Chromiumchloride	108000	64000		1,7	54000	0,8
Coppersulfate	180	20		9	90	4,5
Copperchloride	825000	12000		68	412500	34,4
Leadnitrate	43000	124		364	21500	173,4
Mercurychloride	93	9		10	46,5	5,2
Nicklechloride	6700	15000	0,4		3350	0,2
Zinknitrate	868000	2000000		4,3	434000	2,2
Zinkchloride	93800	10200		9	46900	4,5
Organochlorine insecticides						
Aldrin	33	17		2	16,5	0,9
Aramite	12000	60000	0,2		600	0,01
Dieldrin	79	640	0,1		39,2	0,06
Endrin	50	6		8	25	4,2
Methoxychlor	0,8	0,9	0,8		0,4	0,4
Tetradifon	140	110		1,2	70	0,6
Toxaphene	10	30	0,3		5	0,2
Organophosphate insecticides						
Azinphos methyl	1	0,1		10	0,5	5
Dichlorphos	770	0,5		1540	385	770
Diazinon	1	10	0,1		0,5	0,05
Chlorpyrifos	0,25	0,3	0,8		0,125	0,42
Dimethoate	2900	200		14,5	1450	7,25
Dioxathion	1	8	0,12		0,5	0,06
Disulfoton	100	240	0,4		50	0,2
Ethion	60	2		30	30	15
Fenthion	6	9	0,6		3	0,3
Imidan	6	2		3	3	1,5
Parathion	7	0,6		11,6	3,5	5,8
Phorate	31	5		6,2	16,5	3,1
Phosphamidon	15	16	0,9		7,5	0,45
Temephos	10	82	0,12		5	0,06
Trichlorophon	180	50		3,6	90	1,8
Carbamate insecticides						
Aminocarb	190	12		15,8	95	7,9
Baygon	29	40	0,7		15	0,35
Carbaryl	60	20		30	30	15
Further Pesticides						
Atrazine	6900	5700		1,2	3450	0,6
Bensulide	92000	3300		27,8	46000	13,9
Dicamba	110000	5800		18,9	55000	9,4
Dichlone	14	2		7	7	3,5
Diuron	1000	1000		1	500	0,5
2-4-D	184000	76000		2,4	9200	1,2
Endothall	223000	100000		2,23	111500	1,1
Eptam	14000	35000	0,4		7000	0,2

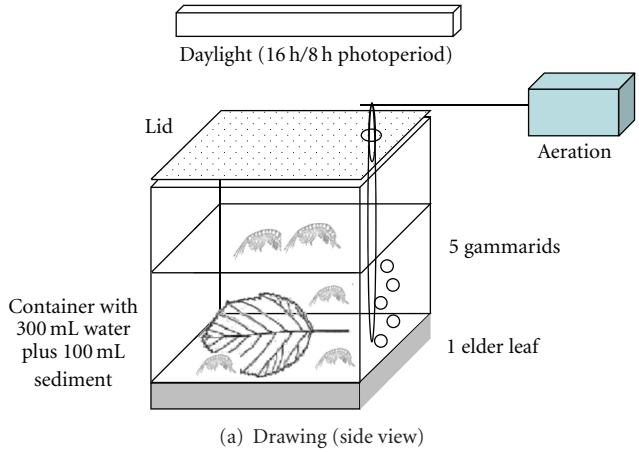
Table 1: Continued.

Substance (μg/l)	Daph-48	Gam-96	Daph/G	D/Gam	Daph-96 (2)	D/G-corr
Molinate	25000	7600		3,2	1250	1,6
Paraquat	6100	18000	0,3		3050	0,1
Picloram	68000	48000		1,4	3400	0,7
Propanil	5000	34000		1,5	2500	0,75
Silvex BEE	7000	740		9,4	3500	4,7
Simazine	100000	21000		4,7	50000	2,3
3.4 DCA	1000	17000	0,05		500	0,025
Pirimicarb	40	48	0,8		20	0,4
Allethrin	20	11		1,8	10	0,9
Pyrethrum	500	12		42	250	21
Terbutryn	2600	4000	0,6		1300	0,3
Fenoxycarb	600	4000	0,15		300	0,075
Lindan	1600	125		12,8	800	6,4
Deltamethrin	0,3	0,005		60	0,15	30
Tebuconazole	4200	1600		2,6	2100	1,3
Imidacloprid	85000	270		314,8	42500	157,4

altered abundance of the species in the ecosystem [11]. 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.



(a) Drawing (side view)

(b) Photograph (view from the top, photo A. Gerhardt)

Figure 1: Experimental unit for the *ex situ* test: (a) drawing, (b) photograph.

2. Methods

2.1. Origin of the Test Organisms and Culture. Gammarus spp. can be collected in a reference stream, being rather unpolluted, in all size classes throughout the year. Chemical water quality parameters and biological bioassessment data ("good water quality" according to EU-WRRL) might be available from routine biomonitoring programmes by the

Figure 2: Experimental setup *in situ* (Photo: A. Gerhardt). View from top: four test chambers (15 × 5 cm) are placed on the sediment of the stream in flow direction, attached with ropes to steel poles in the sediment and on the banks. Each chamber contains 10 gammarids and 2 elder leaves. The test chambers can be connected to the MFB.

water authorities for the choice of appropriate reference conditions. Field-taken organisms need to be acclimated to laboratory test conditions for at least one week prior to testing.

Gammarus spp, taken from the field, might also be maintained during several weeks or even cultured in aerated aquaria with unfiltered stream water for several months. Successful cultures have been described in several previous papers (e.g., [19–21]).

In order to simplify the 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 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 pump with filter (Eheim aquaball 2400, 45 L) is placed in the aquarium within a net cage (0.1 mm mesh size) for water circulation and removal of high loads of particles. Every week the 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-polyethylen 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 sceletize 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 quadrapole impedance conversion technique that simultaneously records several behavioral parameters of a wide range of aquatic organisms, for example, different

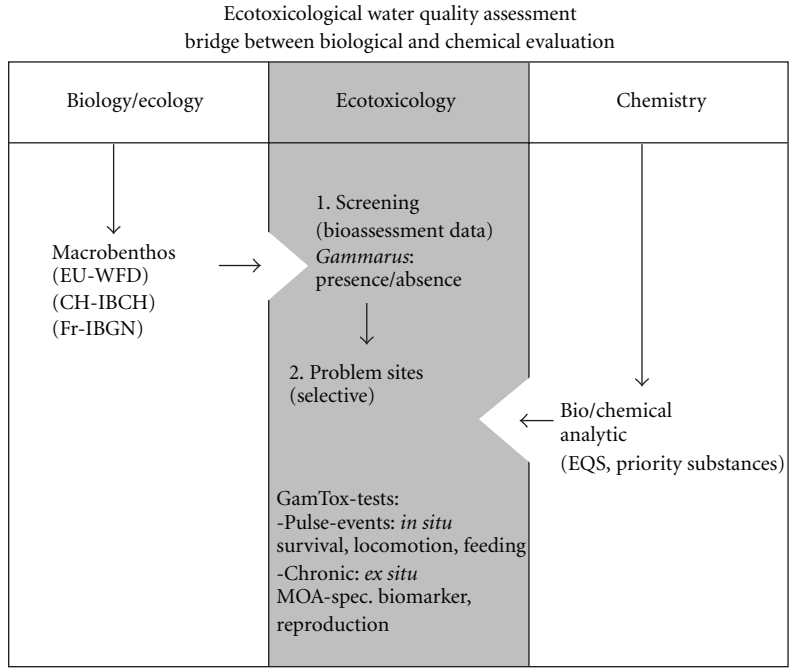


Figure 3: Integrated concept for water quality assessment with a new ecotoxicological module.

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 $AgCl_2$-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 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 traid-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 saproby class 2 (beta-mesosaprob), corresponding to "good" water quality according to the EU-WFD, the decline or presence/absence of gammarids could be a fist 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

[1] R. Vaniola, J. D. S. Witt, M. Grabowski, J. H. Bradbury, K. Jazdzewski, and B. Sket, "Global diversity of amphipods (Amphipoda: Crustacea) in freshwater," *Hydrobiologia*, vol. 595, pp. 155–241, 1979.

[2] C. Macneil, J. T. A. Dick, and R. W. Elwood, "The trophic ecology of freshwater *Gammarus spp* (Crustacea: Amphipoda): problems and perspectives concerning the functional feeding group concept," *Biological Reviews of the Cambridge Philosophical Society*, vol. 72, no. 3, pp. 349–364, 1997.

[3] G. Shaw, "Prey selection by breeding dippers," *Bird Study* , vol. 26, pp. 66–67, 1979.

[4] K. W Cummins and M. J Klug, "Feeding ecology of stream invertebrates," *Annual Review of Ecology, and Systematics*, vol. 10, pp. 147–172, 1979.

[5] N. J. Fielding, C. MacNeil, J. T. A. Dick, R. W. Elwood, G. E. Riddell, and A. M. Dunn, "Effects of the acanthocephalan parasite *Echinorhynchus truttae* on the feeding ecology of *Gammarus pulex* (Crustacea: Amphipoda)," *Journal of Zoology*, vol. 261, no. 3, pp. 321–325, 2003.

[6] Tolosa A. J. Garmendia and B. Axelsson, "*Gammarus*, their biology, sensitivity and significance as test organisms," Tech. Rep. B 1095, Swedish Environmental Research Institute, Stockholm, Sweden, 1993.

[7] J. T. Parsons and G. A. Surgeoner, "Effect of exposure time on the acute toxicities of permethrin, fenitrothion, carbaryl and carbofuran to mosquito larvae," *Environmental Toxicology and Chemistry*, vol. 10, no. 9, pp. 1219–1227, 1991.

[8] EPA, "Gammarid acute toxicity test," 850.1020 guideline. EPA 712-C-96-130, 1996, http://www.epa.gov.

[9] M. C. Fossi, "Biomarkers as diagnostic and prognostic tools for wildlife risk assessment: integrating endocrine-disrupting chemicals," *Toxicology and Industrial Health*, vol. 14, no. 1-2, pp. 291–309, 1998.

[10] A. Gerhardt, "Aquatic behavioral ecotoxicology—prospects and limitations," *Human and Ecological Risk Assessment*, vol. 13, no. 3, pp. 481–491, 2007.

[11] A. Gerhardt, "Monitoring behavioural responses to metals in *Gammarus pulex* (L.) (Crustacea) with impedance conversion," *Environmental Science and Pollution Research*, vol. 2, no. 1, pp. 15–23, 1995.

[12] A. Gerhardt, A. Carlsson, C. Ressemann, and K. P. Stich, "New online biomonitoring system for *Gammarus pulex* (L.) (Crustacea): *in situ* test below a copper effluent in South Sweden," *Environmental Science and Technology*, vol. 32, no. 1, pp. 150–156, 1998.

[13] O.G. Dell, *Behavioral Eecotoxicology*, John Wiley & Sons, New York, NY, USA, 2002.

[14] B. Xuereb, E. Lefèvre, J. Garric, and O. Geffard, "Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): linking AChE inhibition and behavioural alteration," *Aquatic Toxicology*, vol. 94, no. 2, pp. 114–122, 2009.

[15] B. Xuereb, A. Chaumot, R. Mons, J. Garric, and O. Geffard, "Acetylcholinesterase activity in *Gammarus* fossarum (Crustacea Amphipoda). intrinsic variability, reference levels, and a reliable tool for field surveys," *Aquatic Toxicology*, vol. 93, no. 4, pp. 225–233, 2009.

[16] H. J. De Lange, M. Lürling, B. Van Den Borne, and E. T. H. M. Peeters, "Attraction of the amphipod *Gammarus pulex* to water-borne cues of food," *Hydrobiologia*, vol. 544, no. 1, pp. 19–25, 2005.

[17] L. Maltby, S. A. Clayton, R. M. Wood, and N. McLoughlin, "Evaluation of the *Gammarus pulex in situ* feeding assay as a

biomonitor of water quality: robustness, responsiveness, and relevance," *Environmental Toxicology and Chemistry*, vol. 21, no. 2, pp. 361–368, 2002.

[18] M. C. Bloor and C. J. Banks, "An evaluation of mixed species *in situ* and *ex situ* feeding assays: the altered response of *Asellus aquaticus* and *Gammarus pulex*," *Environment International*, vol. 32, no. 1, pp. 22–27, 2006.

[19] M. C. Bloor, C. J. Banks, and V. Krivtsov, "Acute and sublethal toxicity tests to monitor the impact of leachate on an aquatic environment," *Environment International*, vol. 31, no. 2, pp. 269–273, 2005.

[20] C. P. McCahon and D. Pascoe, "Culture techniques for three freshwater macroinvertebrate species and their use in toxicity tests," *Chemosphere*, vol. 17, no. 12, pp. 2471–2480, 1988.

[21] C. P. McCahon and D. Pascoe, "Use of *Gammarus pulex* (L.) in safety evaluation tests: culture and selection of a sensitive life stage," *Ecotoxicology and Environmental Safety*, vol. 15, no. 3, pp. 245–252, 1988.

[22] A. Gerhardt, E. Svensson, M. Clostermann, and B. Fridlund, "Monitoring of behavioral patterns of aquatic organisms with an impedance conversion technique," *Environment International*, vol. 20, no. 2, pp. 209–219, 1994.

[23] A. Gerhardt, L. Janssens De Bisthoven, and A. M. V. M. Soares, "Effects of acid mine drainage and acidity on the activity of *C* (Ephemeroptera: Leptophlebiidae)," *Archives of Environmental Contamination and Toxicology*, vol. 48, no. 4, pp. 450–458, 2005.

[24] L. Janssens De Bisthoven, A. Gerhardt, and A. M. V. M. Soares, "Behavioral changes and acute toxicity to the freshwater shrimp *Atyaephyra desmaresti* millet (Decapoda: Natantia) from exposure to acid mine drainage," *Ecotoxicology*, vol. 15, no. 2, pp. 215–227, 2006.

[25] A. Gerhardt, "Recent trends in online biomonitoring for water quality control," in *Biomonitoring of Polluted Water*, A. Gerhardt, Ed., vol. 9, pp. 95–118, Trans Tech Publications Ltd, Zürich, Germany, 1999.

[26] A. Gerhardt, *Ökotoxikologische Tests an ARA-Abläufen und Fliessgewässern-AquaPlus*, Im Auftrag des Amtes für Umwelt des Kt, St. Gallen, Switzerland, 2010.

[27] A. Gerhardt, *Gammarus spp. Rückgang Oder Fehlen von Bachflohkrebsen in Bächen. AquaPlus*, Department Bau, Verkehr und Umwelt, Kanton Aargau, CH, 2010.

[28] A. Gerhardt, PY. Kunz, C. Kienle, and D. Tropiano, "GamTox—a proposal for a new integrative multimetric aquatic toxicity assessment," in *Abstract and poster presented at PRIMO (Pollutant responses in Marine organisms)*, Bordeaux, France, May 2009.

[29] C. Kienle, D. Tropiano, M. Langer-Jaesrich et al., "Toxicity evaluation of a pulp deposit with test organisms of different trophic levels," in *Abstract and poster presentation at SETAC Europe*, Seville, Spain, May 2010.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 328749, 9 pages
doi:10.1155/2011/328749

Research Article

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

Melanie J. Dixon and Peter J. Shaw

Centre for Environmental Sciences, Faculty of Engineering and the Environment, University of Southampton, Highfield, Southampton SO17 1BJ, UK

Correspondence should be addressed to Peter J. Shaw, ps@soton.ac.uk

Received 23 December 2010; Accepted 8 March 2011

Academic Editor: Michelle Bloor

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 ($1\,\mu$L/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 re-formation 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.

Precopular 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 precopular 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 precopular 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 used 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. pulex* 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 precopular 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. Precopular Re-Exposure Test. A series of re-exposure tests was also conducted to elucidate responses of precopular 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 precopular 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 precopular 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

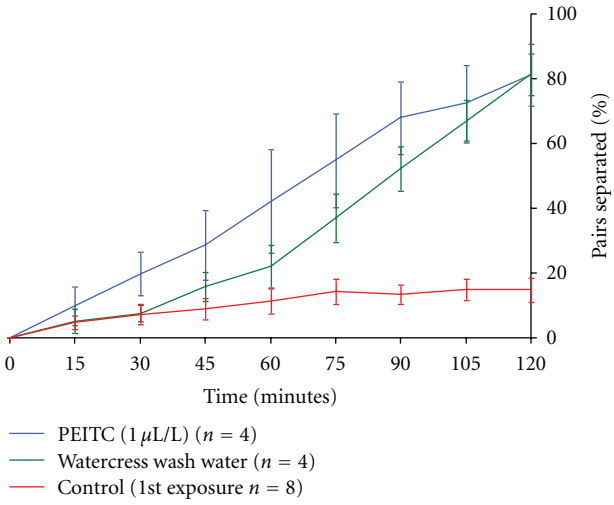


Figure 1: Mean cumulative proportion of pairs separated (±SE). Precopular pairs were exposed at Time = 0, the mean of 4 tests over the course of the 2 h exposure is shown for each test substance (carried out on separate occasions, with a total of 119 pairs exposed to wash water, 105 pairs exposed to 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.

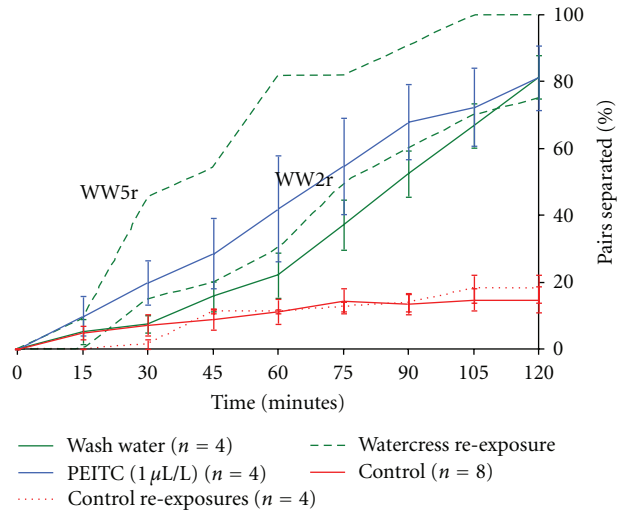


Figure 2: Cumulative proportion of pairs separated-watercress wash water re-exposures. Solid lines show the mean response for four separate initial exposures. The initial wash water exposure (green) is emboldened for comparison with re-exposures (green-dotted lines) to wash water on 2 separate test occasions; Test WW5r (11 pairs) after exposed test organisms had spent 24 h in clean water, and Test WW2r (20 pairs) after exposed test organisms had spent 48 h in clean water. The mean response of 4 control re-exposures (red) follows a similar pattern to the initial control exposure (red-dotted line shows the mean of 8 control exposures). Vertical bars show standard error; n denotes the number of tests.

Table 1: Summary of ET_{50} values. The proportional data were arcsine square root transformed and the ET_{50} calculated using maximum-likelihood probit or logit analysis. WW1–WW5: samples of watercress wash water. P1–P5: samples of PEITC solution.

Sample	ET_{50} (minutes)	95% confidence intervals
WW1	77*	73–85
WW2	106*	103–110
WW3	89	78–102
WW5	84	72–93
P1	48	38–56
P2	119	108–133
P3	85	77–92
P5	40	20–56

* 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 ET_{50} (95% CI) values for pairs re-exposed to watercress wash water were 87 (77–106) and 41 (27–51) minutes. The ET_{50} (95% CI) values for pairs re-exposed to PEITC solution were 54 (41–64) and 40 (19–53) minutes. For all re-exposures, the ET_{50} 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 re-forming 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 re-form 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.

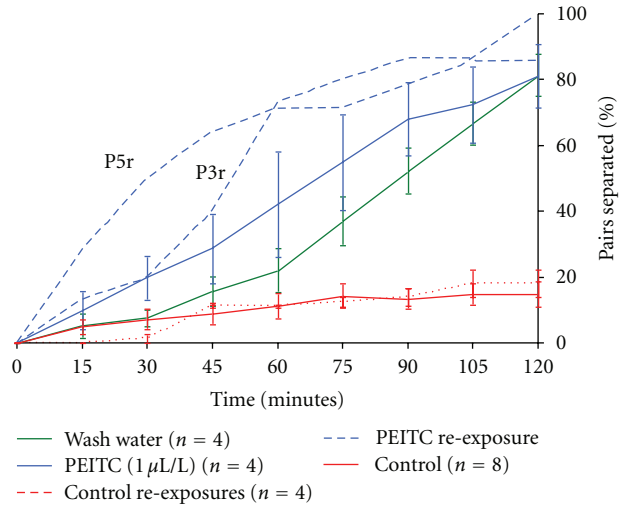


Figure 3: Cumulative proportion of pairs separated-PEITC re-exposures. Solid lines show the mean response for four separate initial exposures. The initial PEITC exposure (blue) is emboldened for comparison with re-exposures (blue-dotted lines) to PEITC on 2 separate test occasions; Test P5r (14 pairs) after exposed test organisms had spent 24 h in clean water, and Test P3r (15 pairs) after exposed test organisms had spent 48 h in clean water. The mean response of 4 control re-exposures (red) follows a similar pattern to the initial control exposure (red-dotted line shows the mean of 8 control exposures). Vertical bars show standard error; n denotes the number of tests.

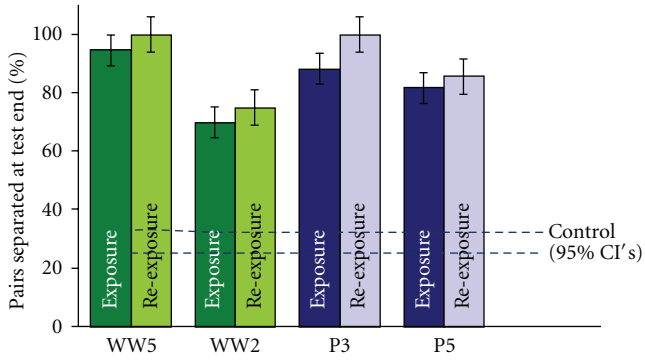


Figure 4: Proportion of pairs separated at two-hour test end (±SE). Wash water exposures (WW5 and WW2) are shown as green, and PEITC exposures (P3 and P5) are shown as blue. The 95% upper and lower confidence intervals for the initial control exposures are shown as dotted lines for comparative purposes.

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

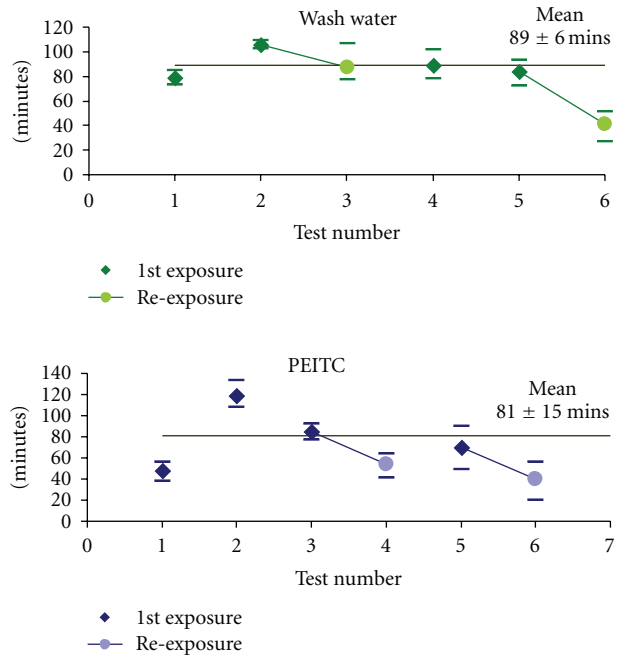


Figure 5: ET_{50} (95% CI) values for exposures and re-exposures. The upper panel shows the ET_{50} value for each PEITC exposure (green diamond) and re-exposure (green circle). The lower panel shows ET_{50} value for each wash water exposure (blue diamond) and re-exposure (blue circle). Re-exposures are linked to the initial exposure by a solid line. Horizontal bars show the 95% upper and lower confidence intervals. Mean ET_{50} (±SE) value for the initial exposures is shown as a solid black line.

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, 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

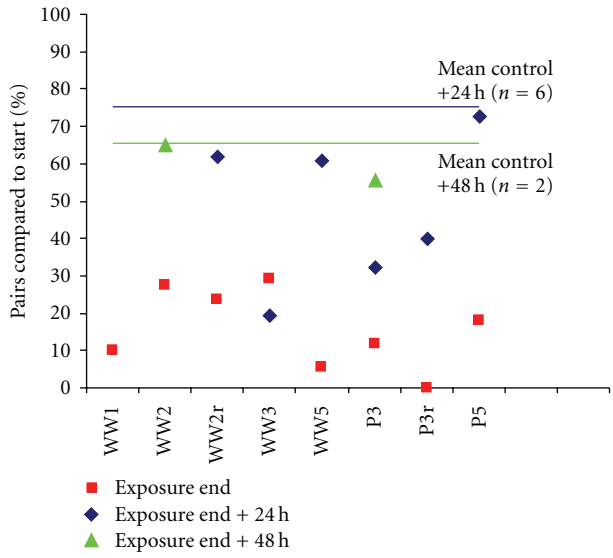


Figure 6: The proportion of pairs re-forming after their return to clean water at exposure end. The proportion of pairs re-forming after transfer to clean water at the initial exposure test end is shown for each separate test occasion. The proportion of pairs present at the start of the first exposure was taken as 100%. The mean control re-pairing is shown for comparison, after 24 h in clean water mean of 6 tests (blue line) and after 48 h in clean water mean of 2 tests (green line). Wash water exposures (WW); PEITC exposures (P); proportion of pairs remaining at initial exposure end (red square); after 24h in clean water (green triangle); after 48 h in clean water (blue diamond). NB: Re-pairing was not recorded after test WW1. *n* denotes the number of tests.

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 ET_{50} 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 moults [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 ET_{50}.

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. pseudolimnaeus* 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 precopular 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 ET_{50} 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

[1] Environment Agency, *The State of England's Chalk Rivers*, UK Biodiversity Action Plan Steering Group for Chalk Rivers Environment Agency, Bristol, UK, 2004.

[2] Hampshire Biodiversity Partnership, *Biodiversity Action Plan for Hampshire*, vol. 2, Hampshire County Council, 2000.

[3] G. Woodward, G. Papantoniou, F. Edwards, and R. B. Lauridsen, "Trophic trickles and cascades in a complex food web: impacts of a keystone predator on stream community structure and ecosystem processes," *Oikos*, vol. 117, no. 5, pp. 683–692, 2008.

[4] A. D. Berrie, "The chalk-stream environment," *Hydrobiologia*, vol. 248, no. 1, pp. 3–9, 1992.

[5] C. P. Mainstone, *Chalk Rivers Nature Conservation and Management*, WRc, 1999.

[6] A. M. Bones and J. T. Rossiter, "The myrosinase-glucosinolate system, its organisation and biochemistry," *Physiologia Plantarum*, vol. 97, no. 1, pp. 194–208, 1996.

[7] J. W. Fahey, A. T. Zalcmann, and P. Talalay, "The chemical diversity and distribution of glucosinolates and isothiocyanates among plants," *Phytochemistry*, vol. 56, no. 1, pp. 5–51, 2001.

[8] R. M. Newman, Z. Hanscom, and W. C. Kerfoot, "The watercress glucosinolate-myrosinase system: a feeding deterrent to caddisflies, snails and amphipods," *Oecologia*, vol. 92, no. 1, pp. 1–7, 1992.

[9] W. C. Kerfoot, R. M. Newman, and Z. Hanscom, "Snail reaction to watercress leaf tissues: reinterpretation of a mutualistic "alarm" hypothesis," *Freshwater Biology*, vol. 40, no. 2, pp. 201–213, 1998.

[10] A. C. Prusak, J. O'Neal, and J. Kubanek, "Prevalence of chemical defenses among freshwater plants," *Journal of Chemical Ecology*, vol. 31, no. 5, pp. 1145–1160, 2005.

[11] A. L. Shelton, "Within-plant variation in glucosinolate concentrations of *Raphanus sativus* across multiple scales," *Journal of Chemical Ecology*, vol. 31, no. 8, pp. 1711–1732, 2005.

[12] J. Brown and M. J. Morra, *Glucosinolate-Containing Seed Meal as a Soil Amendment to Control Plant Pests: 2000–2002*, National Renewable Energy Laboratory, 2005.

[13] R. M. Newman, W. C. Kerfoot, and Z. Hanscom, "Watercress allelochemical defends high-nitrogen foliage against

consumption: effects on freshwater invertebrate herbivores," *Ecology*, vol. 77, no. 8, pp. 2312–2323, 1996.

[14] R. M. Newman, W. C. Kerfoot, and Z. Hanscom, "Watercress and amphipods: Potential chemical defense in a spring stream macrophyte," *Journal of Chemical Ecology*, vol. 16, no. 1, pp. 245–259, 1990.

[15] S. Medgett, *The Impact of St Mary Bourne Cress Farm on the Bourne Rivulet*, Ecological Appraisal Team, Hampshire and Isle of Wight Area Environment Agency, 1998.

[16] H. B. N. Hynes, "The reproductive cycle of some British freshwater Gammaridae," *The Journal of Animal Ecology*, vol. 24, no. 2, pp. 352–387, 1955.

[17] C. P. McCahon and D. Pascoe, "Use of *Gammarus pulex* (L.) in safety evaluation tests: culture and selection of a sensitive life stage," *Ecotoxicology and Environmental Safety*, vol. 15, no. 3, pp. 245–252, 1988.

[18] J. Prenter, C. MacNeil, J. T. A. Dick, G. E. Riddell, and A. M. Dunn, "Lethal and sublethal toxicity of ammonia to native, invasive, and parasitised freshwater amphipods," *Water Research*, vol. 38, no. 12, pp. 2847–2850, 2004.

[19] A. E. Girling, D. Pascoe, C. R. Janssen et al., "Development of methods for evaluating toxicity to freshwater ecosystems," *Ecotoxicology and Environmental Safety*, vol. 45, no. 2, pp. 148–176, 2000.

[20] M. M. Watts, D. Pascoe, and K. Carroll, "Survival and precopulatory behaviour of *Gammarus pulex* (L.) exposed to two xenoestrogens," *Water Research*, vol. 35, no. 10, pp. 2347–2352, 2001.

[21] G. Engelen-Eigles, G. Holden, J. D. Cohen, and G. Gardner, "The effect of temperature, photoperiod, and light quality on gluconasturtiin concentration in watercress (*Nasturtium officinale* R. Br.)," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 2, pp. 328–334, 2006.

[22] U. Palaniswamy, R. McAvoy, and B. Bible, "Supplemental light before harvest increases phenethyl isothiocyanate in watercress under 8-hour photoperiod," *HortScience*, vol. 32, no. 2, pp. 117–205, 1997.

[23] Sigma-Aldrich, "Safety Data Sheet: phenethyl isothiocyanate 253731 (Version 3.0, Revision Date 28/8/09)," in *According to Regulation (EC) 1907/2006*, 2009.

[24] A. Cold and V. E. Forbes, "Consequences of a short pulse of pesticide exposure for survival and reproduction of *Gammarus pulex*," *Aquatic Toxicology*, vol. 67, no. 3, pp. 287–299, 2004.

[25] J. F. C. Malbouisson, T. W. K. Young, and A. W. Bark, "Disruption of precopula in *Gammarus pulex* as a result of brief exposure to gamma-hexachlorocyclohexane (Lindane)," *Chemosphere*, vol. 28, no. 11, pp. 2011–2020, 1994.

[26] E. W. Sexton, "On the rearing and breeding of Gammarus in laboratory conditions," *Journal of the Marine Biological Association of the United Kingdom*, vol. 15, no. 1, pp. 33–56, 1928.

[27] International Organisation for Standardisation, *General requirements for the competence of testing and calibration laboratories*, 2nd edition, 2005.

[28] D. Pascoe, T. J. Kedwards, S. J. Maund, E. Muthi, and E. J. Taylor, "Laboratory and field evaluation of a behavioural bioassay: the *Gammarus pulex* (L.) precopula separation (GaPPS) test," *Water Research*, vol. 28, no. 2, pp. 369–372, 1994.

[29] Vitacress Salads Ltd, *Estimate of Watercress: Wash Water Ratio, Lower Link Farm*, E. Hiscocks, 2008.

[30] Vitacress Salads Ltd, *Crop Wash Statistics May-June 2007 & June-July 2008*, M. Fisher, 2008.

[31] Tidepool Scientific Software, *ToxCalc: Environmental Toxicity Data Analysis System Version 5.0.32*, McInleyville, Calif, USA, 1994.

[32] M. J. Dixon, *The sustainable use of water to mitigate the impacts of watercress farms on chalk streams in Southern England*, Ph.D. thesis, Department of Civil Engineering and the Environment, University of Southampton, 2010.

[33] L. Maltby, S. A. Clayton, R. M. Wood, and N. McLoughlin, "Evaluation of the *Gammarus pulex* in situ feeding assay as a biomonitor of water quality: robustness, responsiveness, and relevance," *Environmental Toxicology and Chemistry*, vol. 21, no. 2, pp. 361–368, 2002.

[34] E. J. Taylor, D. P. Jones, S. J. Maund, and D. Pascoe, "A new method for measuring the feeding activity of *Gammarus pulex* (L)," *Chemosphere*, vol. 26, no. 7, pp. 1375–1381, 1993.

[35] J. S. Welton and R. T. Clarke, "Laboratory studies on the reproduction and growth of the amphipod, *Gammarus pulex* (L.)," *Animal Ecology*, vol. 49, pp. 581–592, 1980.

[36] USEPA, "Gammarid acute toxicity test (OTS-795-120)," in *EPA Test Methods*, 1996.

[37] I. Johnson, M. Hutchings, R. Benstead, J. Thain, and P. Whitehouse, "Bioassay selection, experimental design and quality control/assurance for use in effluent assessment and control," *Ecotoxicology*, vol. 13, no. 5, pp. 437–447, 2004.

[38] Y. Ji, Y. Kuo, and M. E. Morris, "Pharmacokinetics of dietary phenethyl isothiocyanate in rats," *Pharmaceutical Research*, vol. 22, no. 10, pp. 1658–1666, 2005.

[39] V. Gil and A. J. MacLeod, "Degradation of glucosinolates of *Nasturtium officinale* seeds," *Phytochemistry*, vol. 19, no. 8, pp. 1657–1660, 1980.

[40] V. M. Koritsas, J. A. Lewis, and G. R. Fenwick, "Glucosinolate responses of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (*Psylliodes chrysocephala*)," *Annals of Applied Biology*, vol. 118, no. 1, pp. 209–221, 1991.

[41] P. W. Lambdon and M. Hassall, "Do plant toxins impose constraints on herbivores? An investigation using compartmental analysis," *Oikos*, vol. 93, no. 1, pp. 168–176, 2001.

[42] P. Roessingh, E. Städler, G. R. Fenwick et al., "Oviposition and tarsal chemoreceptors of the cabbage root fly are stimulated by glucosinolates and host plant extracts," *Entomologia Experimentalis et Applicata*, vol. 65, no. 3, pp. 267–282, 1992.

[43] K. Rowell and D. W. Blinn, "Herbivory on a chemically defended plant as a predation deterrent in *Hyalella azteca*," *Freshwater Biology*, vol. 48, no. 2, pp. 247–254, 2003.

[44] A. D. P. Worgan and R. Tyrell, "Monitoring behavioural responses of *Gammarus pulex* to watercress oils," Centre for Ecology and Hydrology, Unpublished Report to Vitacress Salads Ltd CEH Project no. 602786NEW, 2005.

[45] Y. Zhang, "Molecular mechanism of rapid cellular accumulation of anticarcinogenic isothiocyanates," *Carcinogenesis*, vol. 22, no. 3, pp. 425–431, 2001.

[46] F. L. Chung, M. A. Morse, K. I. Eklind, and J. Lewis, "Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal," *Cancer Epidemiology Biomarkers and Prevention*, vol. 1, no. 5, pp. 383–388, 1992.

[47] J. W. Chiao, H. Wu, G. Ramaswamy et al., "Ingestion of an isothiocyanate metabolite from cruciferous vegetables inhibits growth of human prostate cancer cell xenografts by apoptosis and cell cycle arrest," *Carcinogenesis*, vol. 25, no. 8, pp. 1403–1408, 2004.

[48] E. A. S. Rosa, "Daily variation in glucosinolate concentrations in the leaves and roots of cabbage seedlings in two constant

temperature regimes," *Journal of the Science of Food and Agriculture*, vol. 73, no. 3, pp. 364–368, 1997.

[49] Environment Agency, *Methods for the examination of waters and associated materials: The Direct Toxicity Assessment of aqueous environmental samples using the juvenile Daphnia magna immobilisation test*, Standing Committee of Analysts, 2007.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 380829, 9 pages
doi:10.1155/2011/380829

Research Article

Imaging and Documenting Gammarideans

Carolin Haug, Gerd Mayer, Verena Kutschera, Dieter Waloszek, Andreas Maas, and Joachim T. Haug

Workgroup Biosystematic Documentation, University of Ulm, Helmholtzstraße 20, 89081 Ulm, Germany

Correspondence should be addressed to Carolin Haug, carolin_haug@gmx.de

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

Academic Editor: Almut Gerhardt

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 450 D 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).

Figure 1: Macrophotographic image of a living gammaridean, *Gammarus roeselii*. An additional Raynox M-150 macroscopic lens was mounted on a Panasonic FZ-50 digital camera. Illumination with two synchronized Nikon SB-20 flashlights.

2.2.2. Documenting Gammarideans Alive or Dead. vDue 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 rerecording 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

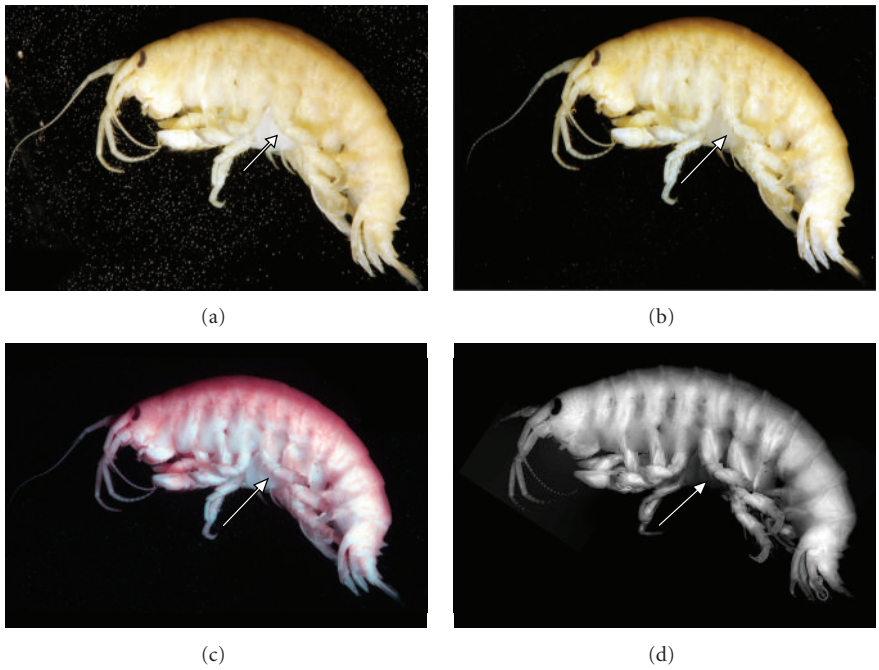


FIGURE 2: Comparison of different methods applied to an entire specimen of *Dikerogammarus villosus* stored in alcohol. All images are fused from several focal planes. (a)–(c) Macrographs. (a) "Normal" macrograph, with optimized uniform light. Bubbles are evident on the black velvet, which was used as 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. Photographing the specimens 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 submersed 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)).

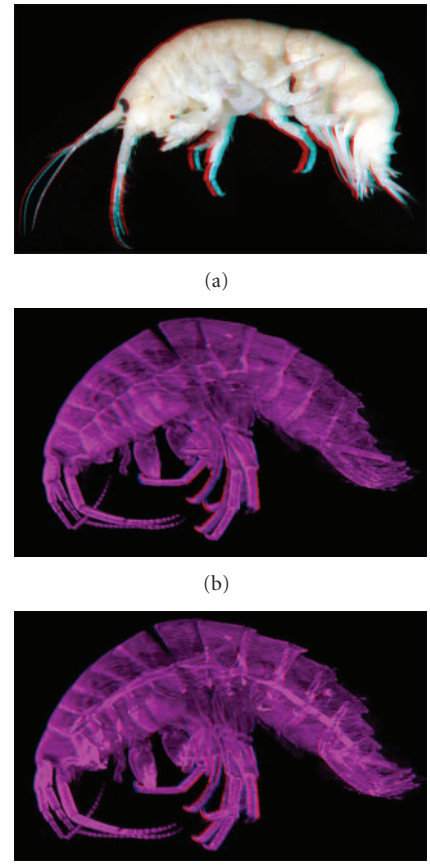
2.2.4. Enhancing the Contrast: Use of Fluorescence. Even stronger contrast than that with crossed polarizing filters can be achieved by the application of fluorescence. Fluorescence photography can be done using a camera or a camera in combination with a fluorescence microscope. In the two cases, the 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).



(a)

(b)

(c)

Figure 3: Stereo images of entire gammaridean specimens (use red-cyan glasses to view). (a) Red-cyan stereo anaglyph based on a pair of macrographs of *Dikerogammarus villosus* recorded under slightly different angles. (b)-(c) Images of *Gammarus roeselii* based on a micro-CT scan. (b) Volume rendering. This presentment emphasizes the outer surface structures. (c) Maximum intensity projection. This presentment enhances the inner structures, as outer surface structures appear transparent.

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

Image Analyzer. Stereo images of the "naked" surface model and of a model with the fused image rendered on it were produced by taking screen shots of the model and processing the images in Adobe Photoshop CS3 to red-cyan anaglyphs. The dark field stack was treated following the procedure described by Haug et al. [15] using ImageJ and OsiriX.

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 endopod 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

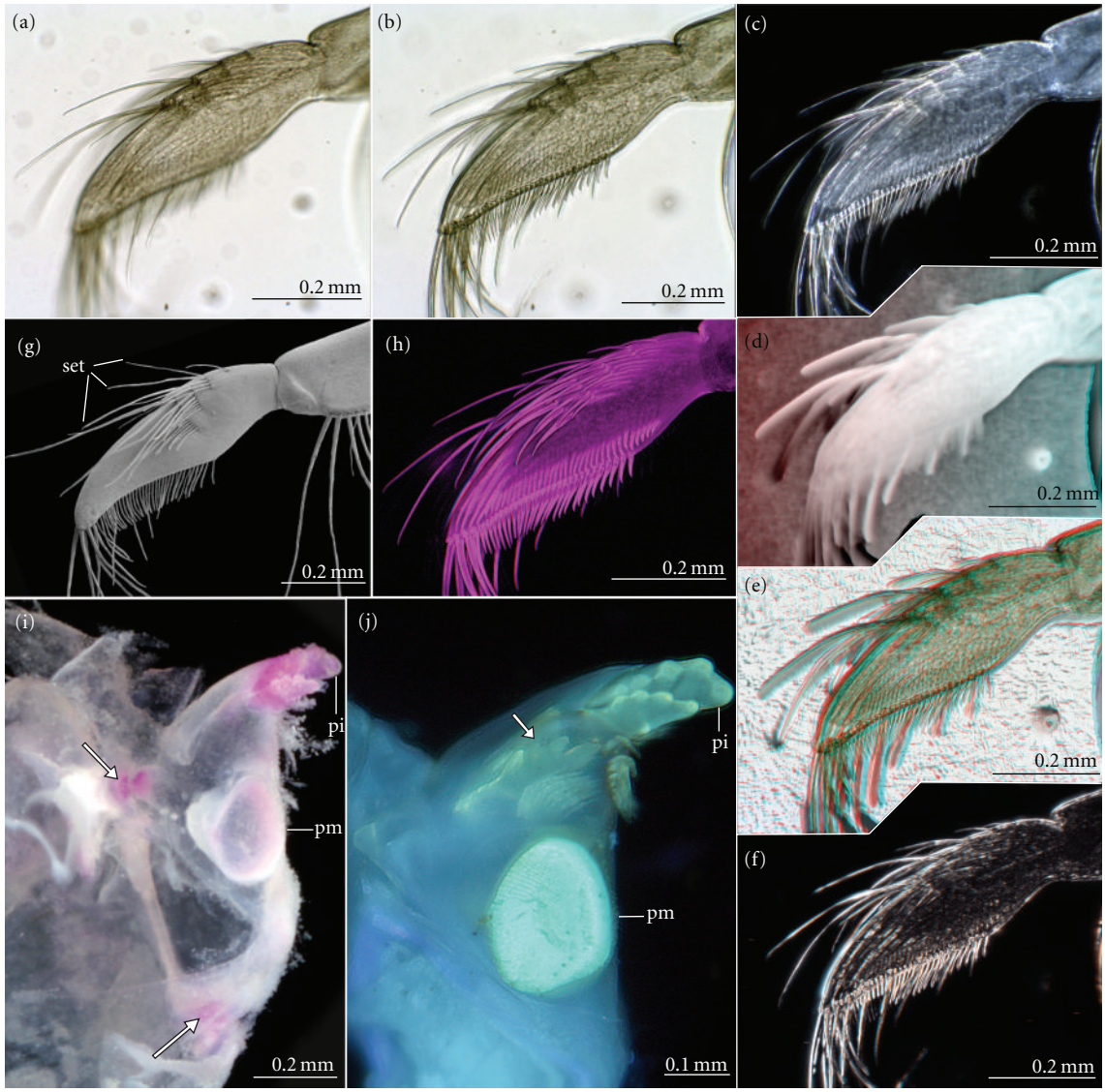


Figure 4: Different microscopical methods applied to structures of the mandible of *Dikerogammarus haemobaphes*. (a)–(h) Tip of the mandibular palp ((a)–(f) and (h) depict the same specimen). Red-cyan glasses needed to experience the stereo effect in (d)–(f) and (h). (a) A single focal plane of a bright 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

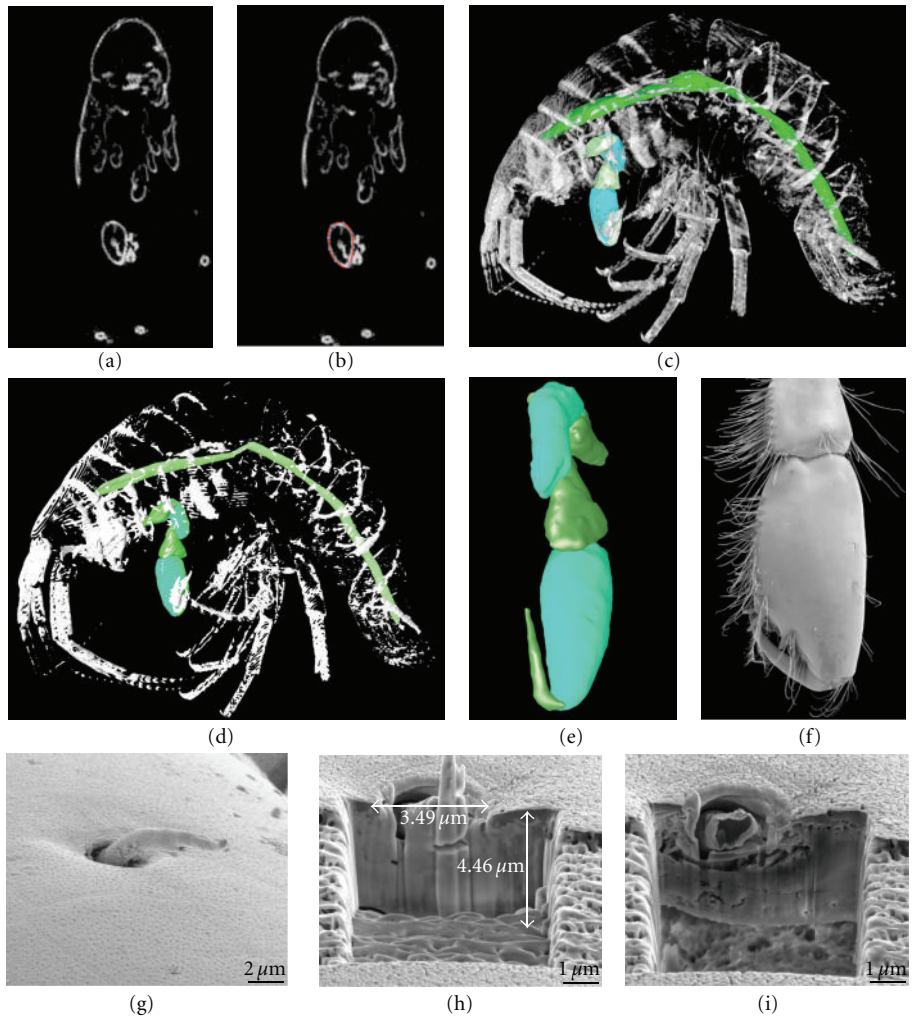


Figure 5: Micro-CT scans ((a)–(d)) and FIB SEM ((g)–(i)). (a) Single virtual slice of a complete microtomogram of a specimen of *Gammarus roeselii.* (b) The same image as (a). The outline of the gnathopod element has been marked in OsiriX (red). (c) Volume rendering of the entire microtomogram. The marked structures are the gnathopod and the gut. (d) Comparable image to (c) but now as a surface model. (e) Surface model of the gnathopod after further processing in the 3D-modelling software Blender. (f) The distal part of a right second gnathopod of *Gammarus roeselii* from posterior as SEM micrograph. (g) A sensillum on the surface of a second gnathopod of *Orchestia cavimana.* (h) Part of the surface close to the sensillum in (g) milled away. (i) Further milling gives access to the inner structure of the sensillum.

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 Mizaikoff, and Christine Kranz, the Institute for Molecular Genetics and Cellular Biology, especially Thomas Gronemeyer, Nils Johnsson, 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 MacNeil, 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

[1] C. S. Wirkner and L. Prendini, "Comparative morphology of the hemolymph vascular system in scorpions—a survey using corrosion casting, MicroCT, and 3D-reconstruction," *Journal of Morphology*, vol. 268, no. 5, pp. 401–413, 2007.

[2] A. V. Klaus, V. L. Kulasekera, and V. Schawaroch, "Three-dimensional visualization of insect morphology using confocal laser scanning microscopy," *Journal of Microscopy*, vol. 212, no. 2, pp. 107–121, 2003.

[3] J. Michels, "Confocal laser scanning microscopy: using cuticular autofluorescence for high resolution morphological imaging in small crustaceans," *Journal of Microscopy*, vol. 227, no. 1, pp. 1–7, 2007.

[4] J. T. Haug, C. Haug, and M. Ehrlich, "First fossil stomatopod larva (Arthropoda: Crustacea) and a new way of documenting Solnhofen fossils (Upper Jurassic, Southern Germany)," *Palaeodiversity*, vol. 1, pp. 97–110, 2008.

[5] T. A. Hegna, "Photography of soft-bodied crustaceans via drying, whitening, and splicing," *Journal of Crustacean Biology*, vol. 30, no. 3, pp. 351–356, 2010.

[6] F. Dellaert, S. M. Seitz, C. E. Thorpe, and S. Thrun, "Structure from motion without correspondence," in *Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition (CVPR '00)*, pp. 557–564, June 2000.

[7] C. Haug, J. T. Haug, D. Waloszek, A. Maas, R. Frattigiani, and S. Liebau, "New methods to document fossils from lithographic limestones of Southern Germany and Lebanon," *Palaeontologia Electronica*, vol. 12, no. 3, article 6T, 2009.

[8] T. Hörnschemeyer, R. G. Beutel, and F. Pasop, "Head structures of *Priacma serrata* Leconte (Coleoptera, Archostemata) inferred from X-ray tomography," *Journal of Morphology*, vol. 252, no. 3, pp. 298–314, 2002.

[9] J. T. Haug, C. Haug, V. Kutschera et al., "Autofluorescence imaging, an excellent tool for comparative morphology," *Journal of Microscopy*, accepted.

[10] D. Platvoet, Z.-E. Hou, S. Li, and G. van der Velde, "Enigmatic pores in females of *Dikerogammarus villosus* (Sowinsky, 1894) (Amphipoda). Amphipod Pilot Species Project (AMPIS) report 1," *Crustaceana*, vol. 78, no. 11, pp. 1399–1404, 2006.

[11] D. Platvoet, Y. Song, S. Li, and G. van der Velde, "A lock-on system in precopulae of *Dikerogammarus villosus* (Sowinsky, 1894), also present in *Gammarus pulex pulex* (Linnaeus, 1758) (Amphipoda). Amphipod Pilot Species Project (AMPIS) report 2," *Crustaceana*, vol. 79, no. 8, pp. 993–1003, 2006.

[12] G. Mayer, G. Maier, A. Maas, and D. Waloszek, "Mouthparts of the Ponto-Caspian invader *Dikerogammarus villosus* (Amphipoda: Pontogammaridae)," *Journal of Crustacean Biology*, vol. 28, no. 1, pp. 1–15, 2008.

[13] G. Mayer, G. Maier, A. Maas, and D. Waloszek, "Mouthpart morphology of *Gammarus roeselii* compared to a successful invader, *Dikerogammarus villosus* (Amphipoda)," *Journal of Crustacean Biology*, vol. 29, no. 2, pp. 161–174, 2009.

[14] S. Bengtson, "Teasing fossils out of shales with cameras and computers," *Palaeontologia Electronica*, vol. 3, no. 1, article 4, 2000.

[15] J. T. Haug, C. Haug, A. Maas, S. R. Fayers, N. H. Trewin, and D. Waloszek, "Simple 3D images from fossil and Recent micromaterial using light microscopy," *Journal of Microscopy*, vol. 233, no. 1, pp. 93–101, 2009.

[16] A. Brösing, S. Richter, and G. Scholtz, "The foregut-ossicle system of *Dromia wilsoni*, *Dromia personata* and *Lauridromia intermedia* (Decapoda, Brachyura, Dromiidae), studied with a new staining method," *Arthropod Structure and Development*, vol. 30, no. 4, pp. 329–338, 2002.

[17] M. Watanabe, S. K. Nayar, and M. Noguchi, "Real-time computation of depth from defocus," in *Three-Dimensional and Unconventional Imaging for Industrial Inspection and Metrology*, vol. 2599 of *Proceedings of SPIE*, pp. 14–25, The International Society for Optical Engineering, Philadelphia, PA, USA, 1995.

[18] S. Chaudhuri and A. Rajagopalan, *Depth from Defocus: A Real Aperture Imaging Approach*, Springer, Heidelberg, Germany, 1999.

[19] V. P. Namboodiri and S. Chaudhuri, "On defocus, diffusion and depth estimation," *Pattern Recognition Letters*, vol. 28, no. 3, pp. 311–319, 2007.

[20] C. Kamenz, J. A. Dunlop, G. Scholtz, H. Kerp, and H. Hass, "Microanatomy of Early Devonian book lungs," *Biology Letters*, vol. 4, no. 2, pp. 212–215, 2008.

[21] B. E. Felgenhauer, "Techniques for preparing crustaceans for scanning electron microscopy," *Journal of Crustacean Biology*, vol. 7, pp. 71–76, 1987.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 248790, 6 pages
doi:10.1155/2011/248790

Research Article

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

Lídia Delgado,[1] Guillermo Guerao,[2] and Carles Ribera[1]

[1] *Institut de Recerca de la Biodiversitat (IRBio) y Departament de Biologia Animal, Universitat de Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain*
[2] *IRTA, Unitat de Cultius Aquàtics (UCA), 43540 Sant Carles de la Ràpita, Tarragona, Spain*

Correspondence should be addressed to Guillermo Guerao, guillermo.guerao@irta.cat

Received 8 March 2011; Accepted 14 April 2011

Academic Editor: Chris Lloyd Mills

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 μm d^{-1} and 13.5 μm d^{-1} at 40 psu and 2 psu, respectively, with the higher values of 18.0 μm d^{-1} and 18.5 μm 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, and 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 the osmoregulatory ability in some species of amphipods [6, 29–32], whereas in *G. aequicauda*, the salinity conditions 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 changed every 48 hours.

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_o: $b = 1$) 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:

$$\text{Log}_{10}\text{BL} = 1.211\log_{10}\text{CL} + 0.2197. \quad (1)$$

(See Figure 1).

The relationship between BL and CL shows positive allometric growth ($b = 1.211$; H_o: $b = 1$; $t = -16.23$; $P < .001$). 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

Table 1: The survival, mean size (CL), mean CL growth rate (GR), and estimated BL growth rate (GR_e) (estimated from mean GR) at 42 d in *Gammarus aequicauda* at different salinity conditions. Abbreviations: AC, acclimation conditions.

AC (psu)	Treatment (psu)	CL ± SD (μm)	GR ± SD (CL, μm d^{-1})	GR_e (BL, μm d^{-1})	Survival (%)	Precopula pairs
34	0	—	—	—	0	—
34	2	—	—	—	0	—
34	4	—	—	—	0	—
34	9	974.6 ± 51	14.9 ± 1.2	120	93.3	+
34	19	1015.2 ± 110	18 ± 2.6	140	88.0	+
34	34	1040.1 ± 36	18.5 ± 1.0	143	90.0	+
34	40	713.7 ± 24	10.9 ± 0.6	80	82.0	—
9	0	—	—	—	0	—
9	2	827.5 ± 50	13.5 ± 1.2	102	78.3	—
9	4	976.9 ± 37	17.3 ± 1.0	132	88.0	+
9	9	924.4 ± 49	16.7 ± 1.2	125	81.6	+

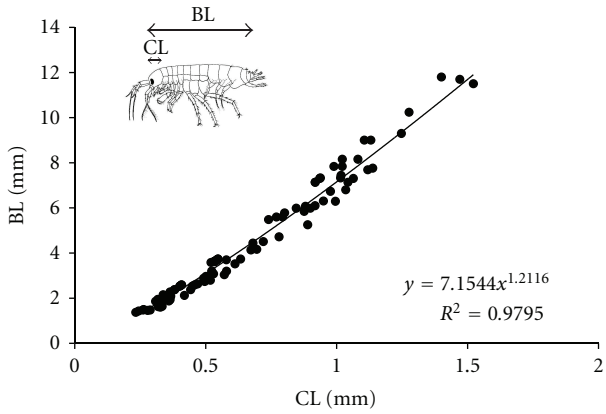


Figure 1: Dispersion diagram of BL (body length in mm) in relation to CL (cephalic length in mm) in 96 individuals of *Gammarus aequicauda*.

(Table 1; Figure 2(a)). At 4 psu, juveniles only survived 16 d. All ovigerous females died after 12–24 h at 0 psu and 2 psu. At 50 psu, brooding females survived, but no juveniles were observed. The size of individuals reared at 40 psu salinity was significantly lower after 42 d than those that received the other treatments ($P < .004$, ANOVA). At salinities between 9 and 34 psu, mature individuals (precopula pairs) were observed at the end of experiment after 42 d of culture. The lower growth rate was 10.9 μm d^{-1}(CL) at 40 psu, and the higher growth rate was 18.5 μm d^{-1}at 34 psu (Figure 3).

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 at 96–108 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 μm 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 cavolinii* Milne-Edwards [38] and the amphipods *Hyale crassicornis* Haswell [39], *Traskorchestia traskiana* Stimpson [40], *Orchesia gammarellus* Pallas [41], *Orchesia chiliensis* Milne-Edwards [42], *Cyathura polita* Stimpson [6, 43] and *G. duebeni* Lillgeborg [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

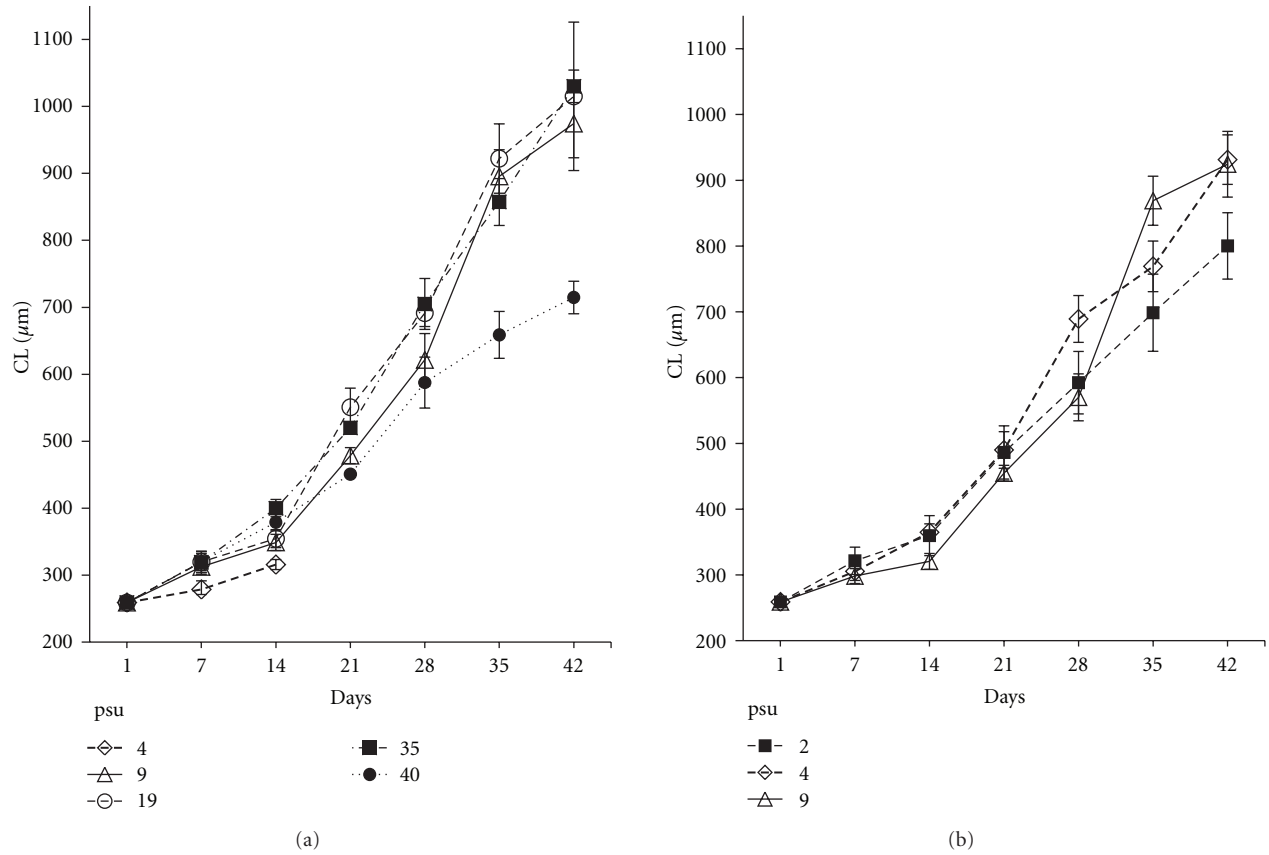


Figure 2: Growth of juveniles of *Gammarus aequicauda* (CL, μm). (a) First experiment: juveniles from ovigerous reproductive adults acclimated at 34 psu. (b) Second experiment: juveniles from reproductive adults acclimated at 9 psu.

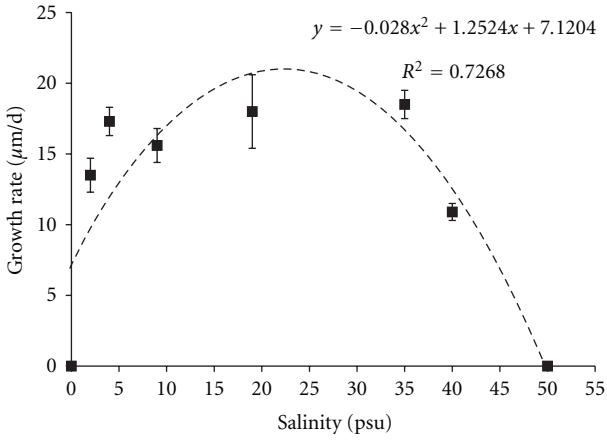


Figure 3: Growth rate of juveniles (CL, $\mu m\, d^{-1}$) of *Gammarus aequicauda* at different tested salinities (0, 2, 4, 9, 19, 34, 40, and 50 psu).

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 $\mu m\, d^{-1}$; estimated from $\log BL = 1.211 \log CL + 0.2197$) are high compared with the values previously recorded by Delgado et al. [13] from their polymodal frequency distribution analyses (21–99 $\mu m\ BL d^{-1}$). 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 recorded 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 lawrencianus* 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

[1] J. Dorgelo, "Comparative ecophysiology of gammarids (Crustacea: Amphipoda) from marine, brackish and fresh-water habitats, exposed to the influence of salinity-temperature combinations - I. Effect on survival," *Hydrobiological Bulletin*, vol. 8, no. 1-2, pp. 90–108, 1974.

[2] J. Dorgelo, "Salt tolerance in crustacea and the influence of temperature upon it," *Biological Review*, vol. 51, pp. 255–290, 1976.

[3] D. H. Steele and V. J. Steele, "Effects of salinity on the survival, growth rate, and reproductive output of *Gammarus lawrencianus* (Crustacea, Amphipoda)," *Marine Ecology Progress Series*, vol. 78, no. 1, pp. 49–56, 1991.

[4] O. Kinne, "Ecological data on the amphipod *Gammarus duebeni*. A monograph," *Veroffentlichungen des Instuts fur Meeresforschungin Bremerhaven*, vol. 6, pp. 177–202, 1959.

[5] O. Kinne, "The effects of temperature and salinity on marine and brackish water animals. 11. Salinity and temperature-salinity combinations," *Oceanography and Marine Biology Annual Review*, vol. 2, pp. 281–339, 1964.

[6] G. Charmantier and M. Charmantier-Öaures, "Ontogeny of osmoregulation in crustaceans: the embryonic phase1," *American Zoologist*, vol. 41, no. 5, pp. 1078–1089, 2001.

[7] E. Cacabelos, A. Lourido, and J. S. Troncoso, "Composition and distribution of subtidal and intertidal crustacean assemblages in soft-bottoms of the Ria De Vigo (NW Spain)," *Scientia Marina*, vol. 74, no. 3, pp. 455–464, 2010.

[8] E. Prato and F. Biandolino, "Seasonal changes in population of the Amphipod *Gammarus aequicauda* (Martynov, 1931)," *Mediterranean Marine Science*, vol. 4, no. 1, pp. 49–56, 2003.

[9] M. Menéndez and F. A. Comín, "Comsumption of macrophytes by invertebrates in Tancada lagoon (NE Spain)," *Scientia Marina*, vol. 54, pp. 139–144, 1990.

[10] I. Petrescu, "Contribution to the knowledge of amphipods (Crustacea) from Romania. II. *Gammarus aequicauda* (Martynov), *G balcanicus* (Chäferna) and *Orchestia cavimana* (Heller)," *Travaux du muséum d'historie naturelle "Grigore Antipa"*, vol. 34, pp. 303–324, 1994.

[11] A. Sfriso, T. Birkemeyer, and P. F. Ghetti, "Benthic macrofauna changes in areas of Venice lagoon populated by seagrasses or seaweeds," *Marine Environmental Research*, vol. 52, no. 4, pp. 323–349, 2001.

[12] C. Casagranda, M. S. Dridi, and C. F. Boudouresque, "Abundance, population structure and production of macroinvertebrate shredders in a Mediterranean brackish lagoon, Lake Ichkeul, Tunisia," *Estuarine, Coastal and Shelf Science*, vol. 66, no. 3-4, pp. 437–446, 2006.

[13] L. Delgado, G. Guerao, and C. Ribera, "The Gammaridea (Amphipoda) fauna in a Mediterranean coastal lagoon: considerations on population structure and reproductive biology," *Crustaceana*, vol. 82, no. 2, pp. 191–218, 2009.

[14] T. Kevrekidis, G. Kourakos, and T. Boubonari, "Life history, reproduction, growth, population dynamics and production of gammarus aequicauda (Crustacea: Amphipoda) at extremely low salinities in a mediterranean lagoon," *International Review of Hydrobiology*, vol. 94, no. 3, pp. 308–325, 2009.

[15] J. T. A. Verhoeven, "The ecology of Ruppia-dominated communities in western Europe. III. Aspects of production, consumption and decomposition," *Aquatic Botany*, vol. 8, no. C, pp. 209–253, 1980.

[16] E. Prato and F. Biandolino, "Amphipod biodiversity of shallow water in the Taranto seas (north-western Ionian Sea)," *Journal of the Marine Biological Association of the United Kingdom*, vol. 85, no. 2, pp. 333–338, 2005.

[17] M. Sezgin and T. Katağan, "An account of our knowledge of the amphipod fauna of the Black Sea," *Crustaceana*, vol. 80, no. 1, pp. 1–11, 2007.

[18] I. I. Greze, "Life cicle of *Gammarus aequicauda* (Martynov, 1931) in the Black Sea," *Crustaceana*, vol. 4, supplement, pp. 88–90, 1977.

[19] T. Kevrekidis and A. Koukouras, "Bionomy of the amphipods in the Evros Delta (north Aegean Sea)," *Marine Ecology*, vol. 9, no. 3, pp. 199–212, 1988.

[20] T. Kevrekidis and A. Koukouras, "Seasonal variation of abundance of *Gammarus aequicauda* (Crustacea: Amphipoda) in the Evros Delta (NE Greece)," *Israel Journal of Zoology*, vol. 36, no. 3-4, pp. 113–123, 1989.

[21] N. Gouvis, T. Kevrekidis, A. Koukouras, and E. Voultsiadou, "Bionomy of macrobenthic polychaetes in the Evros Delta (North Aegean Sea)," *International Review of Hydrobiology*, vol. 83, no. 2, pp. 145–161, 1998.

[22] T. Kevrekidis, A. Mogias, and N. Gouvis, "Interannual changes in the composition of the macrobenthic fauna of Drana lagoon (Evros Delta, n. Aegean Sea): preliminary note," *Belgian Journal of Zoology*, vol. 130, supplement 1, pp. 101–107, 2000.

[23] H. Janssen, M. Scheepmaker, M. Van Couwelaar, and S. Pinkster, "Biology and distribution of *Gammarus aequicauda* and *G. insensibilis* (Crustacea: Amphipoda) in the lagoon system of Bages-Sigean (France)," *Bijdragen tot de Dierkunde*, vol. 49, pp. 42–70, 1979.

[24] E. Prato, F. Biandolino, and C. Scardicchio, "Postembryonic growth, development and reproduction of *Gammarus aequicauda* (Martynov, 1931) (Gammaridae) in laboratory culture," *Zoological Studies*, vol. 45, no. 4, pp. 503–509, 2006.

[25] J. Camp and M. Delgado, "Hidrografía de las bahías del delta del Ebro," *Investigación Pesquera*, vol. 51, pp. 351–369, 1987.

[26] M. Menéndez and F. A. Comín, "Spring and summer proliferation of floating macroalgae in a mediterranean coastal lagoon (Tancada Lagoon, Ebro Delta, NE Spain)," *Estuarine, Coastal and Shelf Science*, vol. 51, no. 2, pp. 215–226, 2000.

[27] M. Menéndez, J. Herrera, and F. A. Comín, "Effect of nitrogen and phosphorus supply on growth, chlorophyll content and tissue composition of the macroalga Chaetomorpha linum (O.F. Müll.) Kütz in a Mediterranean coastal lagoonEfecto De La Adición De Nitrógeno Y Fósforo En El Crecimiento, Concentración De Clorofila Y Nutrientes En La Macroalga Chaetomorpha Linum (O.F. Müll) Kütz En Una Laguna Coostera Mediterránea," *Scientia Marina*, vol. 66, no. 4, pp. 355–364, 2002.

[28] F. A. Comín, "Características físicas y químicas y fitoplancton de las lagunas costeras Encanyissada, Tancada y Buda (Delta del Ebro)," *Oecologia Aquatica*, vol. 7, pp. 79–169, 1984.

[29] N. A. Berezina and V. E. Panov, "Distribution, population structure and salinity tolerance of the invasive amphipod *Gmelinoides fasciatus* (Stebbing) in the Neva Estuary (Gulf of Finland, Baltic Sea)," *Hydrobiologia*, vol. 514, pp. 199–206, 2004.

[30] D. Morritt and J. I. Spicer, "Changes in the pattern of osmoregulation in the brackish water amphipod *Gammarus duebeni* Lilljeborg (Crustacea) during embryonic development," *Journal of Experimental Zoology*, vol. 273, no. 4, pp. 271–281, 1995.

[31] M. F. Gable and R. A. Croker, "The salt marsh amphipod, *Gammarus palustris* Bousfield, 1969 at the northern limit of its distribution. II. Temperature-salinity tolerances," *Estuarine and Coastal Marine Science*, vol. 6, no. 2, pp. 225–230, 1978.

[32] M. Tedengren, M. Arnér, and N. Kautsky, "Ecophysiology and stress response of marine and brackish water Gammarus species (Crustacea, Amphipoda) to changes in salinity and exposure to cadmiun and diesel-oil," *Marine Ecology Progress Series*, vol. 47, pp. 107–116, 1988.

[33] T. Kevrekidis and M. Lazaridou-Dimitriadou, "Relative growth and secondary production of the Amphipod *Gammarus aequicauda* (Martynov, 1931) in the Evros Delta (North Aegean Sea)," *Cahiers de Biologie Marine*, vol. 29, no. 4, pp. 483–495, 1988.

[34] O. Guelorget and J. P. Perthuisot, "Paralic ecosystems: biological organization and functioning," *Vie et Milieu*, vol. 42, no. 2, pp. 215–251, 1992.

[35] M. D. Subida, M. R. Cunha, and M. H. Moreira, "Life history, reproduction, and production of *Gammarus chevreuxi* (Amphipoda:Gammaridae) in the Ria de Aveiro, northwestern Portugal," *Journal of the North American Benthological Society*, vol. 24, no. 1, pp. 82–100, 2005.

[36] P. Maranhão and J. C. Marques, "The influence of temperature and salinity on the duration of embryonic development, fecundity and growth of the amphipod *Echinogammarus marinus* Leach (Gammaridae)," *Acta Oecologica*, vol. 24, no. 1, pp. 5–13, 2003.

[37] G. Charmantier and M. Charmantier Daures, "Ontogeny of osmoregulation and salinity tolerance in the isopod crustacean Sphaeroma serratum," *Marine Ecology Progress Series*, vol. 114, no. 1-2, pp. 93–102, 1994.

[38] M. L. Casabianca, "Influence des apports d'eau douce sur la dynamique des populations de crustacés constructeurs de l'étang de Biguglia, Corse (*Corophium insidiosum* C., *Tanais cavolinii* M. E., *Erichthonius brasiliensis* D.)," *Vie et Milieu*, vol. 23, pp. 45–63, 1973.

[39] K. H. Tsoi, K. M. Chiu, and K. H. Chu, "Effects of temperature and salinity on survival and growth of the amphipod Hyale crassicornis (Gammaridea, Hyalidae)," *Journal of Natural History*, vol. 39, no. 4, pp. 325–336, 2005.

[40] H. Koch, "Salinity tolerance and osmoregulation of *Traskorchestia traskiana* (Stimpson, 1857) (Amphipoda, Talitridae)," *Crustaceana*, vol. 61, no. 1, pp. 21–37, 1991.

[41] P. G. Moore and C. H. Francis, "Environmental tolerances of the beach-hopper *Orchestia gammarellus* (Pallas) (Crustacea:Amphipoda)," *Marine Environmental Research*, vol. 19, no. 2, pp. 115–129, 1986.

[42] I. D. Marsden, "Effects of constant and cyclic temperatures on the salinity tolerance of the estuarine sandhopper *Orchestia chiliensis*," *Marine Biology*, vol. 59, no. 4, pp. 211–218, 1980.

[43] B. J. Kelley and W. D. Burbanck, "Responses of embryonic *Cyathura polita* (stimpson) (Isopoda: Anthuridea) to varying salinities," *Chesapeake Science*, vol. 17, no. 3, pp. 159–167, 1976.

[44] D. W. Sutcliffe and J. Shaw, "Sodium Regulation in the Amphipod *Gammarus duebeni* Lilljeborg from freshwater localities in Ireland," *Journal of Experimental Biology*, vol. 48, pp. 339–358, 1968.

[45] C. Piscart, D. Webb, and J. N. Beisel, "An acanthocephalan parasite increases the salinity tolerance of the freshwater amphipod *Gammarus roeseli* (Crustacea: Gammaridae)," *Naturwissenschaften*, vol. 94, no. 9, pp. 741–747, 2007.

[46] M. Normant, E. Schmolz, and I. Lamprecht, "Heat production rate of the Baltic amphipod *Gammarus oceanicus* at varying salinities," *Thermochimica Acta*, vol. 415, no. 1-2, pp. 135–139, 2004.

[47] T. Neuparth, F. O. Costa, and M. H. Costa, "Effects of temperature and salinity on life history of the marine amphipod *Gammarus locusta*. Implications for ecotoxicological testing," *Ecotoxicology*, vol. 11, no. 1, pp. 61–73, 2002.

Hindawi Publishing Corporation
International Journal of Zoology
Volume 2011, Article ID 598504, 10 pages
doi:10.1155/2011/598504

Research Article

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

Alireza Mirzajani,[1] Mostafa Sayadrahim,[1] and Alireza Sari[2]

[1] *Department of Ecology, Inland Water Aquaculture Research Center, P.O. Box 66, 43167 Guilan, Iran*
[2] *School of Biology, University College of Science, University of Tehran, 14155 Tehran, Iran*

Correspondence should be addressed to Alireza Mirzajani, ar_mirzajani@yahoo.com

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

Academic Editor: Chris Lloyd Mills

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* (Carausu, 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.

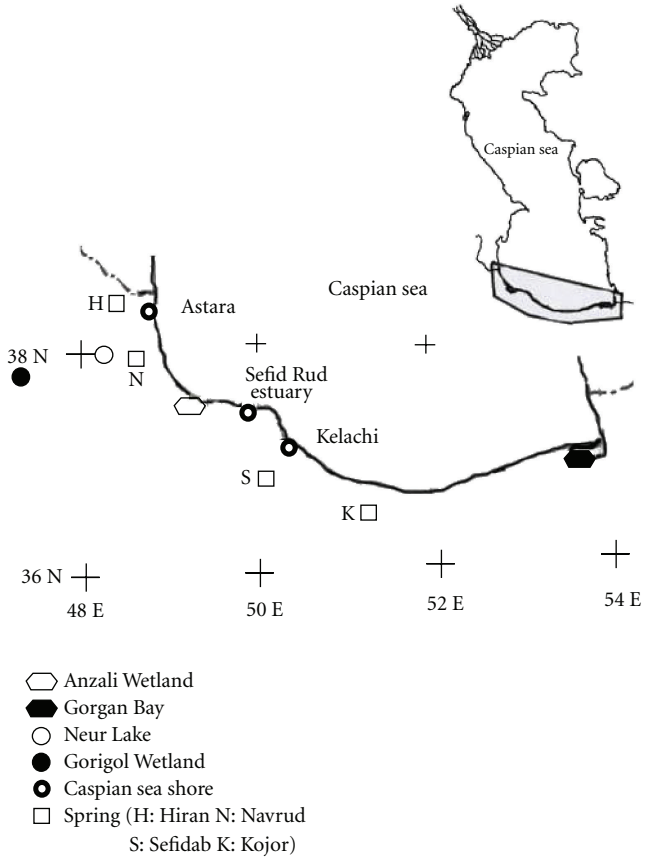


Figure 1: Sampling localities in northern parts of Iran.

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

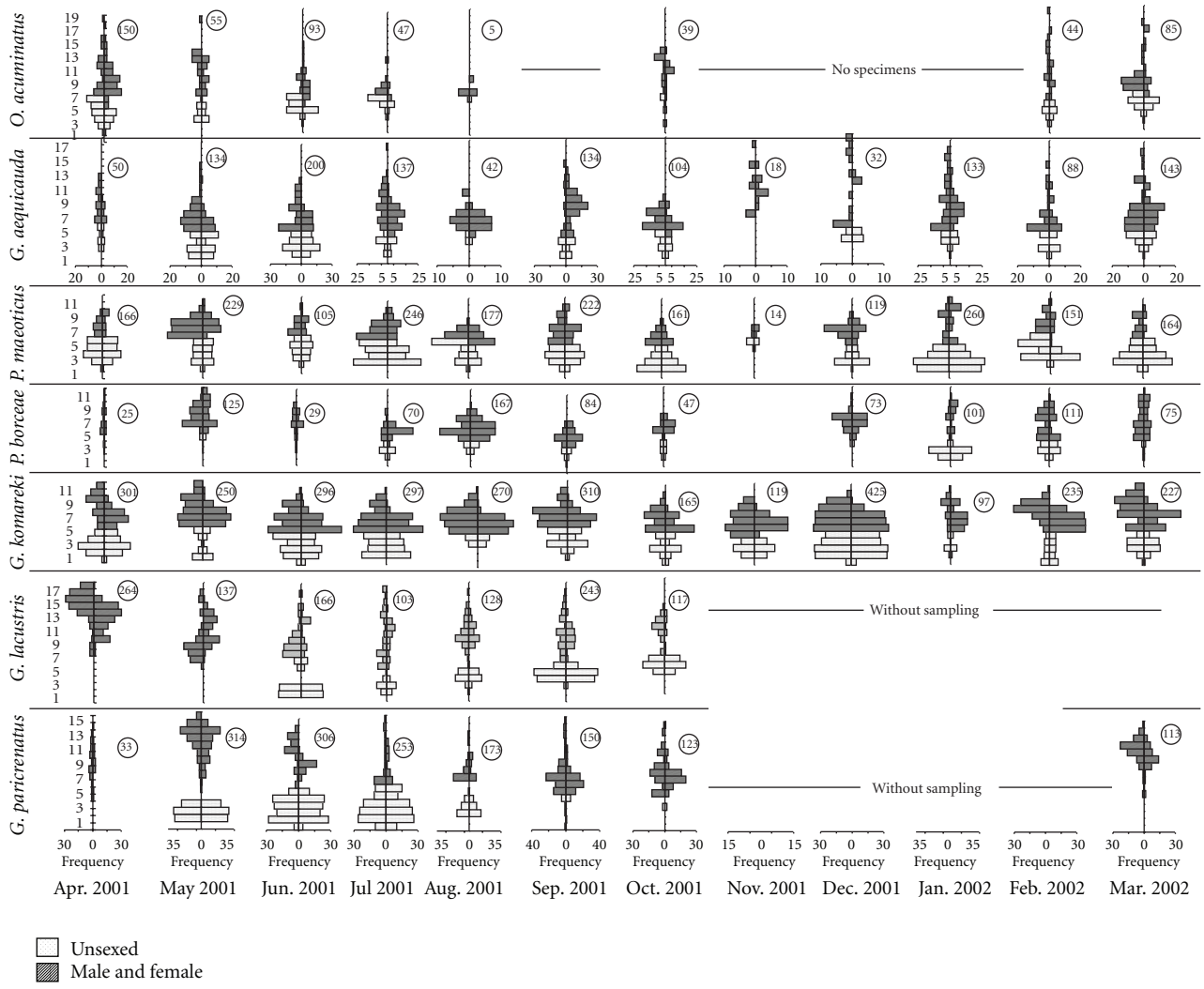


Figure 2: Size and sex composition of studied species during April 2001 to March 2002. The numbers examined are circled.

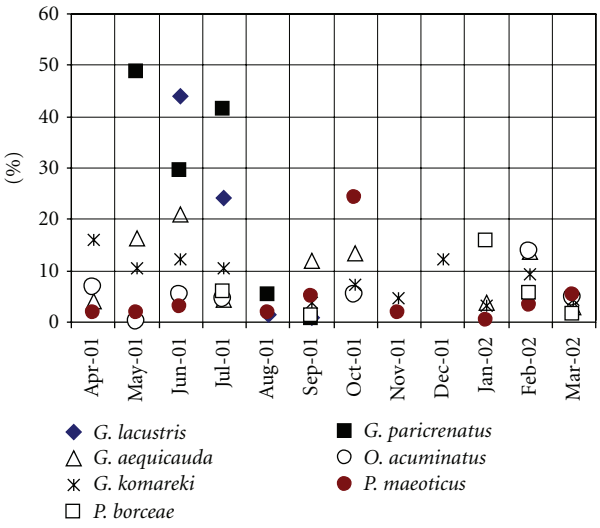


Figure 3: The newly hatched and juvenile (<4 mm long) individuals for each species during April 2001 to March 2002.

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. maeoticus* (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).

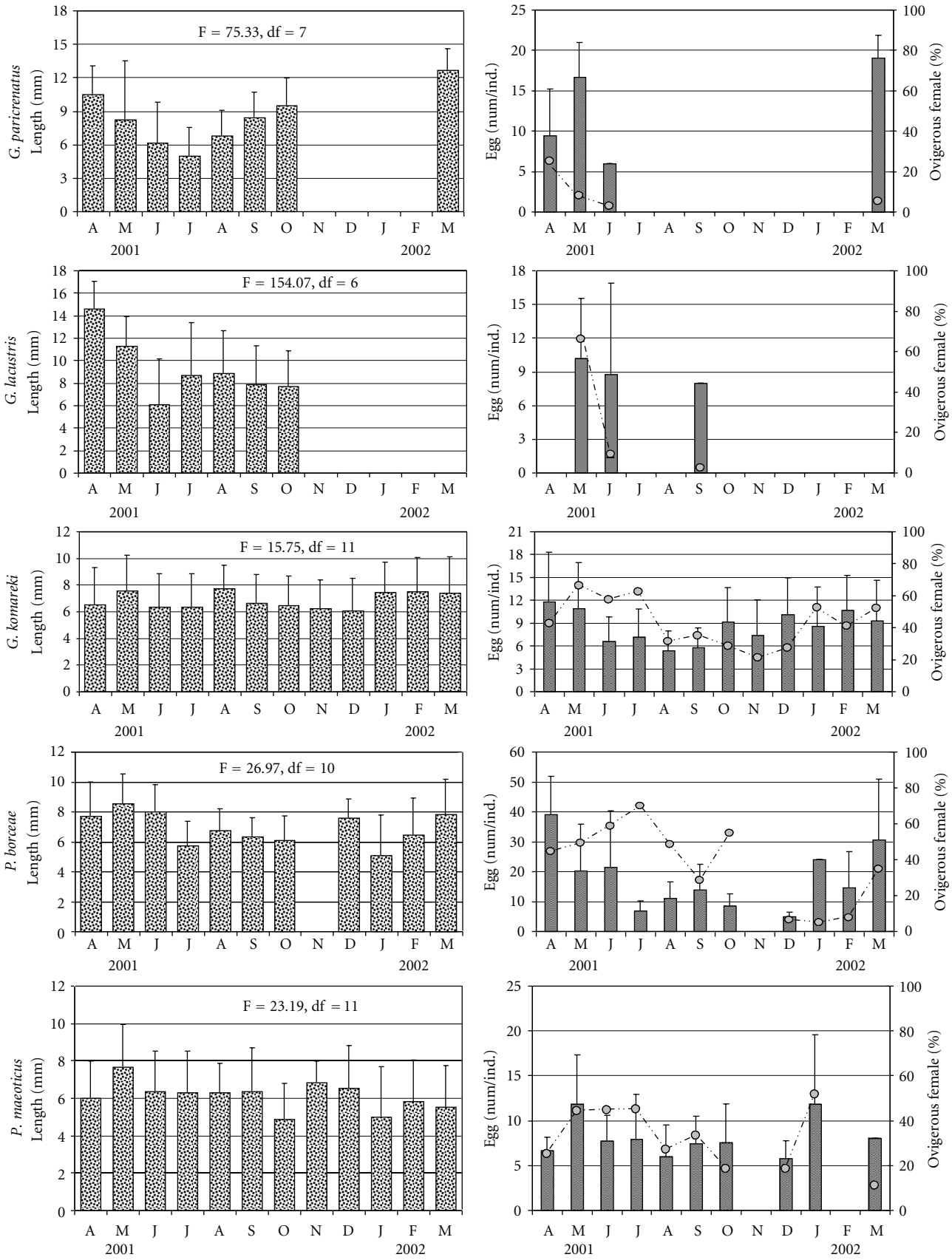


Figure 4: Continued.

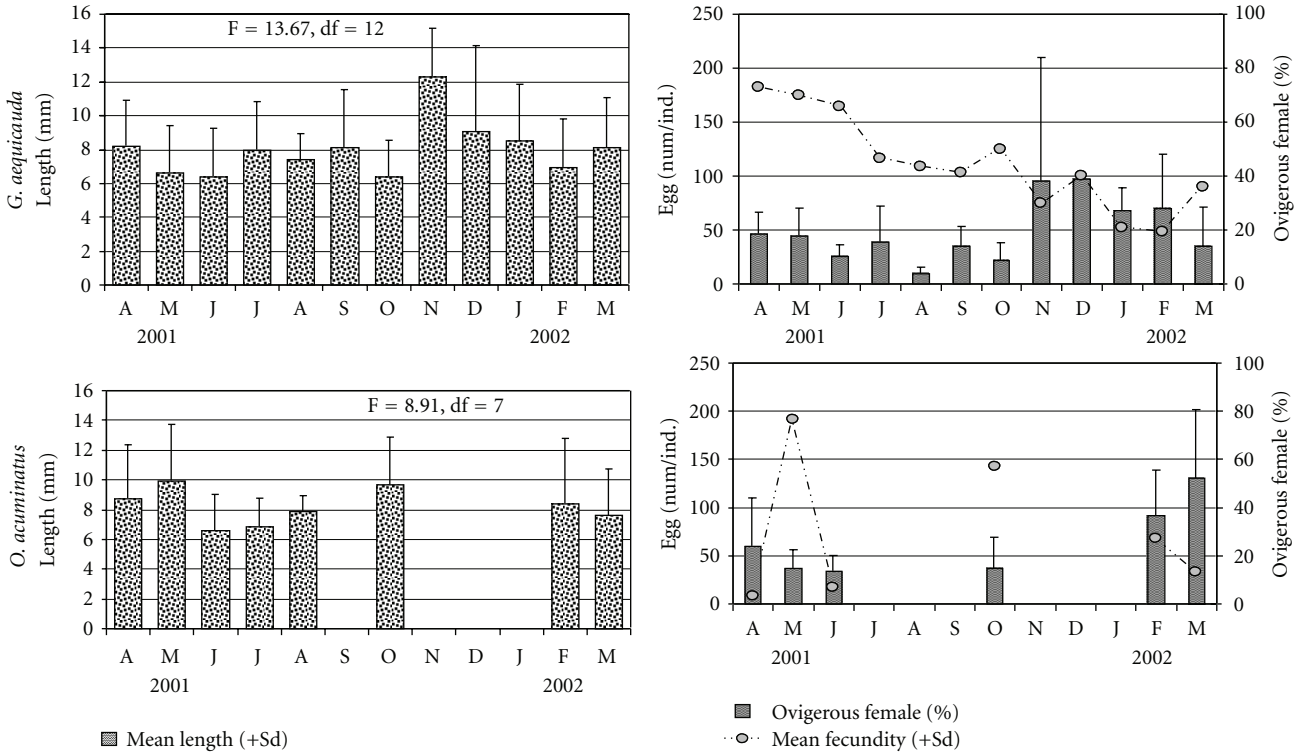


Figure 4: Mean length (mm), fecundity (egg/ind.), and frequency of ovigerous female for each species during April 2001 to March 2002.

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

Species	Sampling site	Position	Altitude	Studied specimens number	Sampling method*
Gammarus lacustris G.O. Sars, 1863	Neur Lake (3 points)	38° 00′ N, 48° 35′ E	2500 m	1158	From substrate by E.d
Gammarus paricrenatus Stock et al. 1998	Gorigol Wetland (3 points)	37° 54′ N, 46° 42′ E	1330 m	1465	From aquatic plant by H.s
Gammarus komareki Schaferna, 1992	Springs; Hiran Navrud Sefidab Kojor	 38° 24′ N, 48° 44′ E 37° 42′ N, 48° 51′ E 37° 00′ N, 50° 18′ E 36° 23′ N, 51° 43′ E	1200 to 1700 m	2924	From stream vegetation and detritus by H.s
Gammarus aequicauda Martynov, 1931	Gorgan Bay (4 points)	36° 50′ N, 53° 45′ E	−10 m	1215	From edge aquatic plant by H.s
Obesogammarus acuminatus Stock et al. 1998	Anzali Wetland (4 points)	37° 29′ N, 49° 21′ E	−5 m	518	From aquatic plant by H.s
Pontogammarus maeoticus (Sowinsky, 1894)	Caspian sea shore; Astara, Sefid Rud estuary Kelachi	 38° 26′ N, 48° 53′ E 37° 27′ N, 49° 55′ E 37° 05′ N, 50° 26′ E	−10 m	2014	From substrate by E.d
Pontogammarus borceae (Carausu, 1943)				907	

* E.d.: Ekman dredge and H.s.: handle sieve.

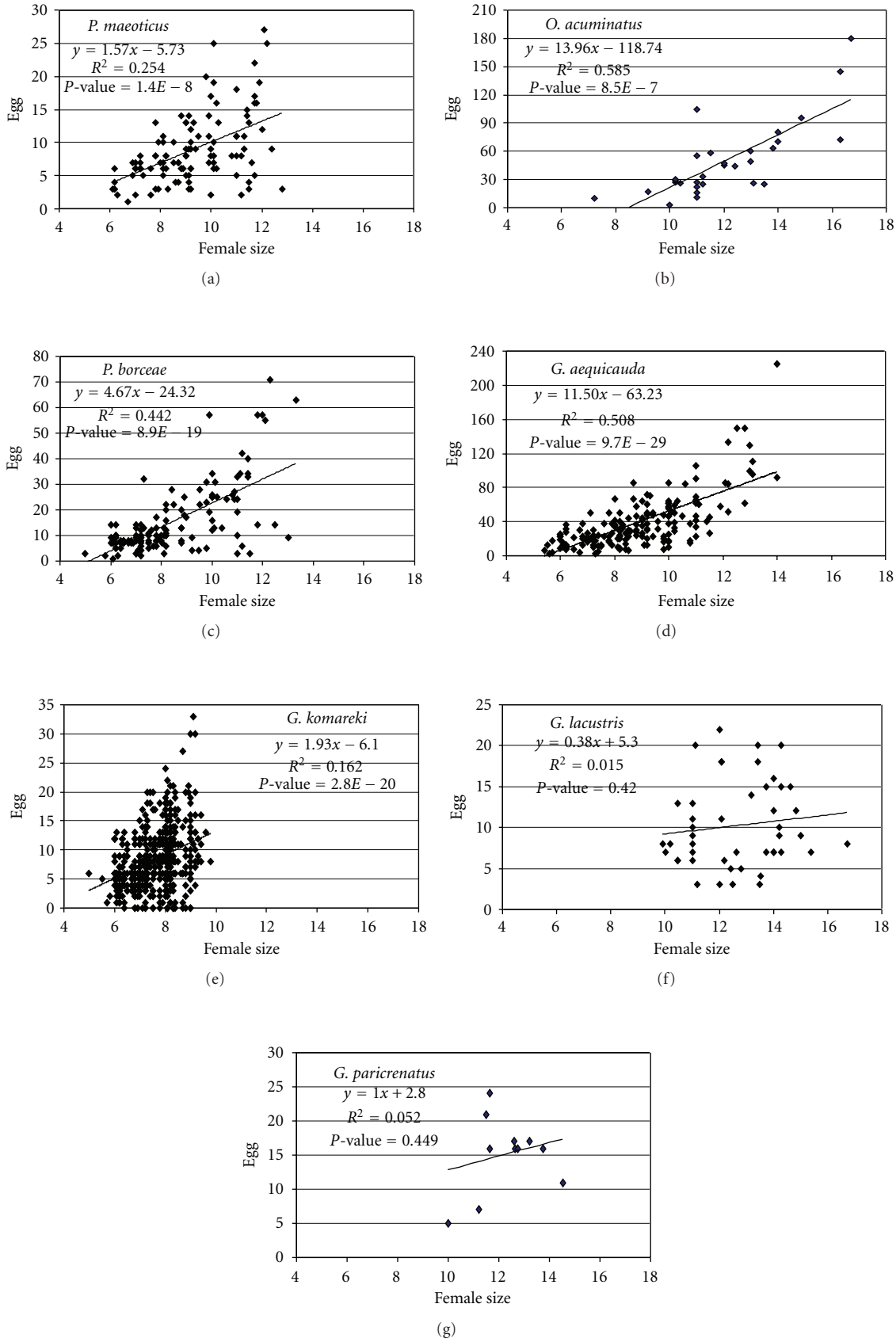


Figure 5: Linear regression of egg number in brood pouch with female size for each species.

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

Species	Mean length ± SE[a]	t-value[b]	Sex ratio (F/M)	Min. and max. length of ovigerous female
Gammarus lacustris G.O. Sars, 1863	$F = 12.42 \pm 0.12$ (321) $M = 12.87 \pm 0.16$ (397)	2.26*	1 : 1.5	8–16.7
Gammarus paricrenatus Stock et al. 1998	$F = 11.01 \pm 0.13$ (274) $M = 12.04 \pm 0.14$ (350)	5.43*	1 : 1.2	10–14.5
Gammarus komareki Schaferna, 1992	$F = 7.65 \pm 0.04$ (876) $M = 8.71 \pm 0.05$ (1093)	16.64*	1 : 1.2	5.5–12
Gammarus aequicauda Martynov,1931	$F = 9.94 \pm 0.11$ (228) $M = 10.94 \pm 0.16$ (274)	6.74*	1 : 1.2	5.4–14
Obesogammarus acuminatus Stock et al. 1998	$F = 11.03 \pm 0.21$ (120) $M = 11.43 \pm 0.26$ (109)	1.17	1 : 1.2	7.2–16.7
Pontogammarus maeoticus (Sowinsky, 1894)	$F = 8.49 \pm 0.09$ (334) $M = 7.89 \pm 0.05$ (641)	5.73*	1 : 2.2	6.1–12.8
Pontogammarus borceae (Carausu, 1943)	$F = 8.62 \pm 0.10$ (236) $M = 8.60 \pm 0.09$ (214)	0.23	1 : 1	5–13.3

[a] The measurements did not include newly hatched and juvenile individuals. The numbers are examined presented in the parenthesis.
[b] t-test for equality of mean length between sexes.
*Significant difference at the .05 level.

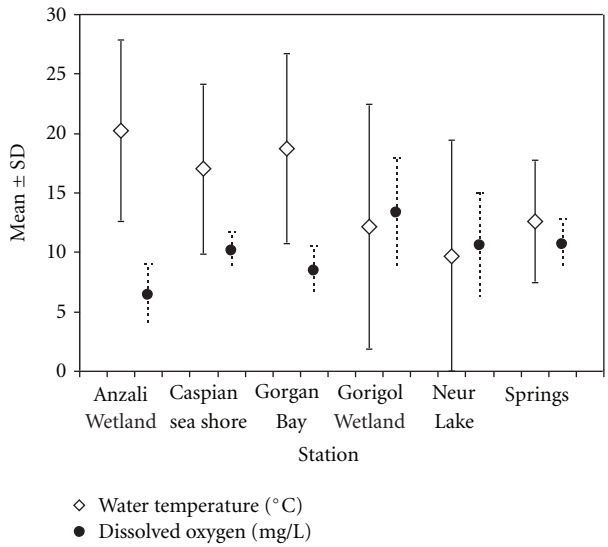


Figure 6: Water temperature and dissolved oxygen in sampling sites during April 2001 to March 2002.

Various lengths of *G. komareki* (Figure 2) were present in all months of the year but the maximum and minimum mean lengths were observed in August and December, respectively (Figure 2). The least percentage of ovigerous females was also distinguished from August to November, increased its trend in the following months, and peaked in spring (Figure 4).

Overall, *G. aequicauda* and *O. acuminatus* showed the highest fecundity with mean egg numbers of 37.7 ± 31.1 and 49.8 ± 39.9, respectively, while *G. komareki* and *P. maeoticus* had a few eggs per female with 8.8 ± 5.0 and 8.8 ± 5.3, respectively. The greatest percentages of ovigerous 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. borceae* was 10.2 ± 5.2 and 14.9 ± 13.1, respectively.

Table 3: Pearson correlation of mean egg number with percentage of ovigerous females during different months for each species.

Species	Correlation value (Sig. level)
G. paricrenatus	−0.22 (0.78)
G. lacustris	0.97 (0.17)
G. komareki	0.15 (0.65)
P. borceae	−0.02 (0.96)
P. maeoticus	0.65 (0.04)*
G. aequicauda	−0.46 (0.13)
O. acuminutus	−0.41 (0.42)

* Correlation is significant at the .05 level (2-tailed).

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. borceae* ($r = 0.664$, $P < .01$), 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 Gorigol 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

Table 4: Some abiotic characteristics of the habitats.

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

* From a single sampling during October 2001.

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 (DIP). The springs Hiran, Navrud, and Kojor 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).

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 Jazdzewski [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

mature in the following spring, and in August and September the parents are completely replaced by a new generation [23]. Other species [24], for example, *Gammarus setosus*, that adapted to the low temperatures of northern environments showed a single brood produced per year. According to Sainte-Marie [5], the cold-water animals are lager and live longer but show late maturity. These are breeding once a year and produce few broods during lifetime. As high latitude amphipods can have resting stages, it is possible that the immature specimens of *G. lacustris* and *G. paricrenatus* entered the resting stage directly in autumn and early winter rather than producing a brood immediately.

The reproduction indices of *G. lacustris* and *G. paricrenatus* suggest the K breeding strategy, similar to Arctic and Antarctic benthic crustaceans such as *Onisimus litoralis* that have a long life cycle, larger eggs, and also larger female size [25].

From the habitat characteristics it is understood that high oxygen level is critical for survival of *G. komareki* and Ponto-Caspian species while *G. paricrenatus* prefers habitats with a higher pH, and low salinity. This species is able to tolerate high seasonal temperature and high daily oxygen variations whereas *G. komareki* lives in habitats with very low salinity, weakly alkaline pH and highly oxygenated water (Table 4 and Figure 6). The high values for phosphorus and nitrogen in Neur Lake (Table 4) can be compared with other localities from previous works [26], which showed that *G. lacustris* was present in 53 lakes with pH > 6.3 and absent in highly acid, eutrophic, or polyhumic lakes.

Two other benthos species, *Pontogammarus maeoticus,* and *P. borceae* live in brackish water with salinity of 7.1 ± 4.1 ppt, fine sandy shores, well oxygenated water, and weakly alkaline pH (Table 4). According to Kasymov [27], *P. maeoticus* migrates to a depth of two to three metres when temperatures fall below a certain limit. Ovigerous females begin to appear at 8.2°C in March and begin to move to shallower water (one to two metres) when the temperatures reachd 17.8°C in October in the southern region of the Caspian Sea [27]. In this study small *P. maeoticus* was observed from November to February when the temperature was below 12°C.

The common Mediterranean intertidal species *G. aequicauda* lives in a wide range of salinity between 5.6 and 22.6 ppt in Gorgan Bay. According to Mirzajani et al. [28], the trophic condition is tending towards eutrophic status in Anzali Wetland, which may be a reason for the absence of *O. acuminatus in* our sampling.

The higher brood size of *G. aequicauda* and *O. acuminatus* (Figure 5) is explained by Nelson [3] who reported that female body and brood size were greater in brackish water and epifauna species than in fully freshwater and infauna species. Sainte-Marie [5] likewise pointed out the greater values of number of broods, fecundity, and reproductive potential in brackish water across salinity gradients. The lower values for minimum female size and mean brood size in *G. komareki* (living in spring vegetation and stream substrate detritus) than in *P. maeoticus* and *P. borceae* (burrowing in the soft sand of sublittoral regions) agree with Nelson findings [3]. It seems digging into sand, staying 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.

References

[1] M. D. Subida, M. R. Cunha, and M. H. Moreira, "Life history, reproduction, and production of *Gammarus chevreuxi* (Amphipoda:Gammaridae) in the Ria de Aveiro, northwestern Portugal," *Journal of the North American Benthological Society*, vol. 24, no. 1, pp. 82–100, 2005.

[2] A. A. Vorobyeva and R. S. Nikonova, "Gammarids *Dikerogammarus haemobaphes* (Eichwald) and *Niphagoides maeoticus* (Sowinsky) as aquaculture species," *Hydrobiological Journal*, vol. 23, no. 6, pp. 52–56, 1987.

[3] W. G. Nelson, "Reproductive pattern of Gammaridean amphipods," *Sarsia*, vol. 65, no. 2, pp. 61–71, 1980.

[4] S. Kolding and T. M. Fenchel, "Pattern of reproduction in different population of five species of the amphipod genus *Gammarus*," *Oikos*, vol. 37, pp. 167–172, 1981.

[5] B. Sainte-Marie, "A review of the reproductive bionomics of aquatic gammaridean amphipods: variation of life history traits with latitude, depth, salinity and superfamily," *Hydrobiologia*, vol. 223, no. 1, pp. 189–227, 1991.

[6] A. R. Mirzajani, "A biological study of Gammaridae (Amphipoda) In the South Caspian Sea Basin (Iranian water) for use in fish culture," Registration 83.180, Agricultural Research and Education Organization, 2004.

[7] J. H. Stock, A. R. Mirzajani, R. Vonk, S. Naderi, and B. H. Kiabi, "Limnic and brackish water Amphipoda (Crustacea) from Iran," *Beaufortia*, vol. 48, no. 8, pp. 163–224, 1998.

[8] A. R. Mirzajani, "A study on population biology of *Pontogammarus maeoticus* (Sowinsky, 1894) in Bandar Anzali, southwest Caspian Sea," *Zoology in the Middle East*, vol. 30, pp. 61–68, 2003.

[9] A. R. Mirzajani and B. H. Kiabi, "Distribution and abundance of coastal Caspian Amphipoda (Crustacea) in Iran," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 511–516, 2000.

[10] P. H. D. Mordukhai-Boltovskoi, "Composition and distribution of Caspian fauna In the light of modern data," *Internationale Revue Gesamten Hydrobiologie*, vol. 64, no. 1, pp. 1–38, 1979.

[11] S. Carausu, E. Dobreanu, and C. Manolache, "Amphipoda forme salmastre si de apa dulce Fauna Republicii populare Romine," *Crustacea*, vol. 4, no. 4, pp. 1–409, 1955.

[12] G. Karaman and G. S. Pinkster, "Freshwater *Gammarus* species from Europe, North Africa and adjacent region of Asia (Crustacea-Amphipoda)—part I. *Gammarus* pulex-group and related species," *Bijdragen tot de Dierkunde*, vol. 47, pp. 1–79, 1977.

[13] G. M. Pjatakova and A. G. Tarasov, "Caspian Sea amphipods: biodiversity, systematic position and ecological peculiarities of some species," *International Journal of Salt Lake Research*, vol. 5, no. 1, pp. 63–79, 1996.

[14] J. A. Birstein, "Zametka o presnovodnyhk visshikh rakoobrazykh Turkemenii I Irana," *Uchenye zapiski Moskovskogo Gosudarstvennogo Universiteta*, vol. 83, pp. 151–164, 1945.

[15] A. Y. Yemelyanova, T. A. Temerova, and A. G. Degermendzhy, "Distribution of *Gammarus lacustris* Sars (Amphipoda, Gammaridae) in Lake Shira (Khakasia, Siberia) and laboratory study of its growth characteristics," *Aquatic Ecology*, vol. 36, no. 2, pp. 245–256, 2002.

[16] APHA, *Standard Methods for Examining of Water and Waste Water*, Washington, DC, USA, 20th edition, 1998.

[17] D. H. Steele and V. J. Steele, "The biology of *Gammarus* (Crustacea, Amphipoda) in the northwestern Atlantic—I. *Gammarus dubeni* Lillj," *Canadian Journal of Zoology*, vol. 47, pp. 235–244, 1969.

[18] N. J. Alouf, "Ecologie et cycle de reproductive Gammarus du Assi (= Oronte, liban) (Crustacea, Amphipod)," *Annales de Limnologie*, vol. 16, pp. 119–134, 1980.

[19] F. Charfi-Cheikhrouha, M. ElGtari, and M. F. Bouslama, "Distribution and reproduction of two sandhoppers, Talitrus saltator and Talorchestia brito from Zouaraa beach-dune system (Tunisia)," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 621–629, 2000.

[20] D. H. Steele and V. J. Steele, "Some aspect of the biology of *Calliopius laeviusculus* (Kroyer) (Crustacea, Amphipoda) in the northwestern Atlantic," *Canadian Journal of Zoology*, vol. 51, pp. 723–728, 1973.

[21] F. O. Costa and M. H. Costa, "Review of the ecology of *Gammarus locusta* (L.)," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 541–559, 2000.

[22] K. Jazdzewski, "Biology of Crustacea malacostraca in the Bay of Puck, Polish Baltic sea," *Zoologica Poloniae*, vol. 20, pp. 423–480, 1970.

[23] A. Konopacka and M. Bazewicz-Paszkowycz, "Life history of *Synurella ambulans* (F. Müller, 1846) (Amphipoda, Crangonyctidae) from central Poland," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 597–605, 2000.

[24] V. J. Steele and D. H. Steele, "The biology of *Gammarus* (Crustacea, Amphipoda) in the northwestern Atlantic—II. *Gammarus setosus* Dementieva," *Canadian Journal of Zoology*, vol. 48, pp. 659–671, 1970.

[25] J. M. Westawski, K. Opalinski, and J. Legezynska, "Life cycle and production of Onisimus litoralis (Crustacea Amphipoda): The key species in the Arctic soft sediment littoral," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 585–596, 2000.

[26] V. Yakovlev, "Occurrence of *Gammarus lacustris* G.O.Sars (Amphipoda) in the North-eastern Fennoscandia and the Kola Peninsula in relation to natural and anthropogenic factors," *Polskie Archiwum Hydrobiologii*, vol. 47, no. 3-4, pp. 671–680, 2000.

[27] A. G. Kasymov, *Ecology of the Caspian Lake*, Baku, Azerbaijan, 1994.

[28] A. R. Mirzajani, S. H. Khodaparast Sharifi, H. Babaei, A. Abedini, and A. D. Ghandi, "Eutrophication trend of anzali wetland based on 1992–2002 data," *Journal of Environmental Studies*, vol. 35, no. 52, pp. 65–74, 2010.

[29] I. B. Musko, "The life history of *Dikerogammarus haemobaphes* (Eichw.) (Crustacea: Amphipoda) living on macrophytes in lake Balaton (Hungary)," *Archiv für Hydrobiologie*, vol. 127, no. 2, pp. 227–238, 1993.

[30] A. R. Mirzajani, "Adaptation and use of amphipoda in fish culture ponds in order to increase fish production," Registration 2009/1108, Agricultural Research and Education Organization, 2009.