Visualisation of the Copepod Female Reproductive System using Confocal Laser Scanning Microscopy and Two-Photon Microscopy

Author(s): Susan C. Fitzer, John D. D. Bishop, Gary S. Caldwell, Anthony S. Clare, Robert C. Upstill-Goddard and Matthew G. Bentley
Source: Journal of Crustacean Biology, 32(5):685-692. 2012.
Published By: The Crustacean Society
URL: http://www.bioone.org/doi/full/10.1163/193724012X639788

copulation, a spermatophore is often released onto the male fifth pereiopod before placement onto the female copulatory pore (Hopkins, 1978). Discharge of the seminal contents into the female seminal receptacles follows the attachment of the spermatophore, but the process is not well understood. The seminal contents are liquid and foamy in form (Hopkins, 1978) comprising of immotile spermatozoa. The seminal contents are stored for the fertilisation of successive egg batches throughout the remainder of a female's reproductive life.

We have developed a method using the fluorescent dye 4′6′ diamidino-2-phenylindole (DAPI) to examine the internal anatomy of the female copepod urosome using CLSM. A particular focus of this study is to image the seminal receptacles in relation to spermatophore attachment in order to

to overcome these problems. An objective with a longer working distance would potentially relax these constraints, the probable slight reduction in resolution being relatively unimportant at the scale studied.

Confocal Microscopy

The imaging platform was a Zeiss LSM 510 META confocal microscope. UV excitation of DAPI was performed using a MaiTai multiphoton laser at 720 nm with fluorescence detected between 435-485 nm. The MaiTai laser produces two low energy photons, which combine to enable a higher energy excitation at the fluorescent molecule (Denk et al., 1990). The advantage of using two-photon excitation is that it provides three-dimensional contrast and resolution, with the high-energy laser only applied at the focal volume, which is particularly useful for thick specimens, as the signal yield is enhanced. A green HeNe laser (543 nm) was used to observe the internal structure auto-fluorescence emission (565-615 nm). Red auto-fluorescence was overlaid with blue emissions from DAPI-labelled seminal contents. Images were taken in z-stacks from the focused first slice of interest (nearest the overlaying cover slip) to the last slice of interest (nearest the bottom of the Petri dish). All images were observed using a Plan-Neofluar oil emersion $\times 40$ objective lens (numerical aperture 1.3), which enabled internal examination of the urosome whilst also imaging the spermatophore oo368.6(t.1(52688.8(bot((ne‖rest)-368.6(the68678.8(bo(mrest)).21(sligh7(blue)-352.5(emissiran9.7(in396o)14.2(v98

Fig. 3. Female copepods after attachment of a DAPI-labelled spermatophore, seen in both ventral (A, B) and lateral views (C, D) showing the spermatophore(s) (outside urosome) auto-fluorescing in red. A, schematic representation of female copepod indicating location of urosome image; B, pH 8.10 female with spermatophore attached to outside of urosome (slice 2/19); C, schematic representation of female copepod indicating location of urosome image; D, pH 8.10 female two with spermatophore attached externally to urosome (slice 9/19).

N = 3, 58.3 μm $\pm$ 7.6 μm) compared to copepods cultured at pH 8.10 (71.7 μm $\pm$ 2.9 μm) (Fig. 5). Spermatophore width did not differ significantly (pH 7.67 = 34.167 μm $\pm$ 1.443 μm; pH 8.10 = 40.00 μm $\pm$ 4.33 μm; $P = 0.091$) (Fig. 5). Mean female copepod size at stage C5/C6 was determined to examine ratios of spermatophore size relative to copepod size, as males were discarded and

Fig. 4. Female copepods after attachment of a DAPI-labelled spermatophore, seen here in both ventral (A, B) and lateral views (C, D) showing the spermatophore (s) (outside urosome), reproductive system (ro) and longitudinal muscles (lm) (inside urosome), auto-fluorescing in red. A, schematic representation of female copepod indicating location of urosome image; B, pH 7.67 female with DAPI-labelled spermatophore attached to outside of urosome, seen in lateral view (slice 5/30); C, schematic representation of female copepod indicating location of urosome image; D, pH 7.67 female, with DAPI-labelled spermatophore attached to outside of urosome, seen in lateral view (slice 10/30).

DISCUSSION

Demonstration of the Efficacy of DAPI Labelling

The female genital field located internally to the female genital pore has been described in detail (Fahrenbach, 1962) but through diagrams produced following stereomicroscope imaging made possible due to the transparency of the copepods. The CLSM method for internal imaging of the female urosome provided promising results with clear images of the reproductive system using auto-fluorescence. Specimens used to determine spermatophore placement and the process of seminal fluid transfer into the female urosome were effectively labelled. DAPI labelling of the spermatophore and the seminal contents supports the earlier descriptions of the process of spermatophore discharge (Fahrenbach, 1962; Hopkins, 1978). Fahrenbach (1962) suggested the attached spermatophore draws seawater from the surrounding area to aid discharge of its contents into the

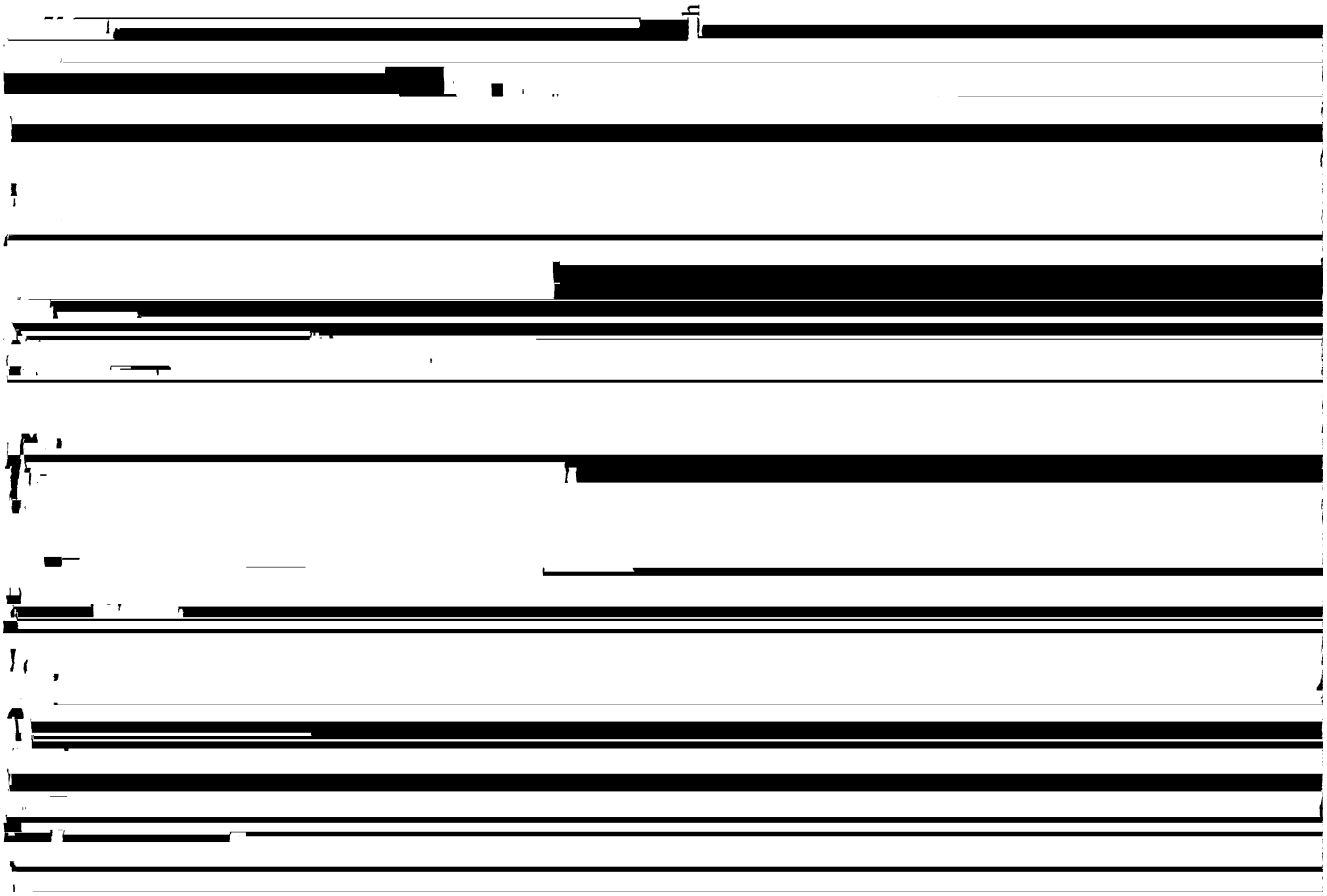

Fig. 5. Copepod spermatophore size differences at two experimental pH levels. A, spermatophore length; B, ratios of spermatophore length to body length; C, spermatophore width. Error bars represent one standard error.

female. However, no images were provided to support this hypothesis. Successful DAPI labelling of spermatophores was achieved in the present study by placing copulating pairs in DAPI solution before spermatophore attachment. This is consistent with the above hypothesis, since DAPI may have been drawn into the spermatophore with the ingress of water following attachment, as the spermatophore released its contents into the female gonopore.

Observation of auto-fluorescent tissues under red light was unexpected but proved beneficial in differentiating the spermatophore contents from the female reproductive system. The tissue auto-flourescence observed under the red filter contrasted against the DAPI fluorescence of the spermatophore contents, observed under a blue filter. Muscles present in the dorsal internal structure of the copepod were clearly visible along with the female reproductive system at pH 8.10 (Fig. 2B, D).

Comments and Recommendations

The use in the present study of two-photon excitation to image the DAPI labelling was not essential, but may have yielded slightly improved resolution compared to single-photon excitation with an ultra-violet laser. However, the two-photon approach creates less cell damage, and provides better tissue penetration (Denk et al., 1990), potentially aiding the imaging deeper into the copepod tissues under the gonopore.

The phalloidin labelling methodology appeared to work; however, due to tissue red-auto-fluorescence it was difficult to determine the extent of labelling. Previous studies have reported difficulties in penetrating the copepod external cuticle when using DAPI and PicoGreen due to the chitinous exterior (Zirbel et al., 2007). Zirbel et al. (2007) used an osmotic gradient in order to label eggs with DAPI whereas Michels (2007) used lactic acid to bleach and clear the cuticle. Lactic acid (Michels, 2007) and HCl proved most useful in the removal of the copepod cuticle for penetration of Texas red-x phalloidin and the removal of external DAPI fluorescence in this study. This study also experienced an initial difficulty in dye penetration, but further confocal analysis of the female copepods revealed internal tissue auto-fluorescence under a red filter. Although the phalloidin staining appeared successful, it was not necessary for observations of internal tissues; auto-fluorescence by itself proved sufficient to ‘map’ the expected seminal fluid stores.

Future application of CLSM to other small crustaceans will require refinement of the spermatophore labelling technique. In particular, in the present study it was only possible to label the spermatophore during copulation in DAPI-spiked seawater.

The differences in spermatophore wall auto-”uorescence with increased acidi“cation was apparently clear-cut, but was only observed for a very limited sample size. This was also the case for the smaller spermatophore size observed with increased acidi“cation (albeit to the same degree as copepod body size). The low sample size was due to dif“culties in obtaining female copepods with attached labelled spermatophores, and further experimentation will be necessary to provide more solid evidence as to the impact of ocean acidi“cation on internal reproductive systems. Further developments in specimen mounting to enable full imaging through the female would enhance the quality of the visual evidence. Even with its current limitations, CLSM can be applied to other zooplankton species of similar size as a means to examine internal reproduction.

ACKNOWLEDGEMENTS

Thanks are due to Dr Glen Wheeler at the Marine Biological Association of the United Kingdom (MBA) for his support during the laser scanning confocal microscopy imaging. Financial support for this research was provided through a NERC-CASE Ph.D. studentship with the MBA to SCF.

REFERENCES

Blades-Eckelbarger, P. I., and M. J. Youngbluth. 1984. The ultrastructure of oogenesis and yolk formation in*Labidocera aestiva*(Copepoda: Calancoida). Journal of Morphology 179: 33-46.

Butcher, R. W. 1959. An Introductory Account of the Smaller Algae of British Coastal Waters. Part I: Introduction and Chlorophyceae, Vol. Ser. IV (Part 1). Ministry of Agriculture Fisheries and Food, Great Britain, 74 pp.

Buttino, I., A. Ianora, Y. Carotenuto, V. Zupo, and A. Miralto. 2003. Use of the confocal laser scanning microscope in studies on the developmental biology of marine crustaceans. Microscopy Research and Technique 60: 458-464.

Denk, W., J. H. Strickler, and W. W. Webb. 1990. Two-photon laser scanning ”uorescence microscopy. Science 248: 73-76.

Eckelbarger, K. J., and P. I. Blades-Eckelbarger. 2005. Oogenesis in calanoid copepods. Invertebrate Reproduction and Development 47: 167-181.

Egilsdottir, H., J. I. Spicer, and S. D. Rundle. 2009. The effect of pCO_2 acidi“ed seawater and reduced salinity on asCgThe ul-30128-344.2(J.)-348.7(Y)(Leach.7(Co3-565.)Tj 7.9(.)-509 4296c [(of)-523.3(oogenesis)-Pol01 (P)1