

# Hormonal induction of undescribed males resolves cryptic species of cladocerans

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Cyclic parthenogens have a mixed breeding system with both meiotic and ameiotic eggs. Although investment in sexual stages is often synchronized with seasonal cycles, the degree of investment is a quantitative trait associated with habitat instability. Populations of cyclic parthenogens from stable environments, such as large lakes and oceans, generally show reduced or undetectable investment in males. Indeed, males of many species of lacustrine cyclic parthenogens are unknown to science. Methyl farnesoate (MF), a crustacean juvenile hormone, has been implicated as an inducer of male formation in *Daphnia magna* (Crustacea: Cladocera), a denizen of unstable habitats with marked sexual recruitment. Here, we show experimentally that MF induces male production in four distantly related lacustrine species of cladocerans under growth conditions unfavourable for male production. The males of three species are new to science. Unlike females, the anatomy of the previously unknown males of *Bosmina* (*Lumobosmina*) *oriens* permitted ready morphological diagnosis of sibling species and subfossils. The results suggest that the role for MF in the sex determination of cladocerans is general.

**Keywords:** cyclic parthenogens; methyl farnesoate; cladoceran; male induction

## 1. INTRODUCTION

Cyclic parthenogenesis (CP) is found in many ecologically successful animal groups, including agricultural pests (aphids) (Moran 1992), and ecological model species (rotifers and cladocerans; Hebert 1978; Wallace & Snell 2001). Although CP has many flavours, populations with this breeding system ordinarily consist entirely of females reproducing via ameiotic eggs. Males and meiotic eggs are infrequently produced, often in association with environmental deterioration (pond drying, salinity changes, starvation, etc.; Dodson & Frey 2001; Benzie 2005). Cyclic parthenogens from stable environments such as large lakes, oceans and tropical waters generally show reduced or undetectable investment in males and sexual females (Brooks 1957). One reason for reduced male production may be that cyclic parthenogen populations can rapidly evolve a reduced male production when the environment is stable and asexual reproduction is favoured (Fussmann *et al.* 2003; Tessier & Cáceres 2004). Absence of males may also be common in lakes because hybrid lineages, which often exhibit reduced male production, can replace sexual parent taxa (Taylor & Hebert 1993a,b; Benzie 2005). Still other CP species may produce males under lake ice (D. J. T. & A. A. K. unpublished observation), which makes detection difficult. The result is that males of many lacustrine species of cyclic parthenogens are poorly studied or unknown.

This dearth of males has stymied scientific study. Males are often a significant source of taxonomic characters in cyclic parthenogens, especially in the Cladocera.

Moreover, secondary sexual characters show weak within-species variation compared to non-sexual characters (Johnson 1952; Ishida *et al.* in press). DNA-based characters have mitigated the species problem, but morphological characters remain critical for understanding and comparing paleolimnological and classical ecological findings (Deevey & Deevey 1971). Also, lines of research that depend on breeding studies such as quantitative genetics, experimental hybridization and genetic mapping are prohibited without males. Finally, the biology of males is largely unstudied in many cyclic parthenogens.

Recent progress has been made in understanding the physiological basis of male production in the microcrustacean cyclic parthenogen, *Daphnia magna* Straus, a species that is generally associated with marked sexual recruitment (Olmstead & LeBlanc 2002, 2003; Tatarazako *et al.* 2003). Specifically, methyl farnesoate (MF), a crustacean juvenile hormone, has been shown to induce male formation during oocyte maturation in *D. magna*. MF is synthesized by the mandibular gland in some crustaceans, and it has been proposed that MF transduces environmental cues for male production in water fleas leading to sex chromosome loss and male formation. Insecticide analogues of juvenile hormone, such as fenoxycarb, have similarly been shown to induce males in daphniids with marked sexual investment (Oda *et al.* 2005).

In the present study, we investigated the effect of MF on the induction of males from distantly related cladoceran families. We examined lacustrine species with a typically reduced investment in males (three of the four taxa have males that are unknown to science).

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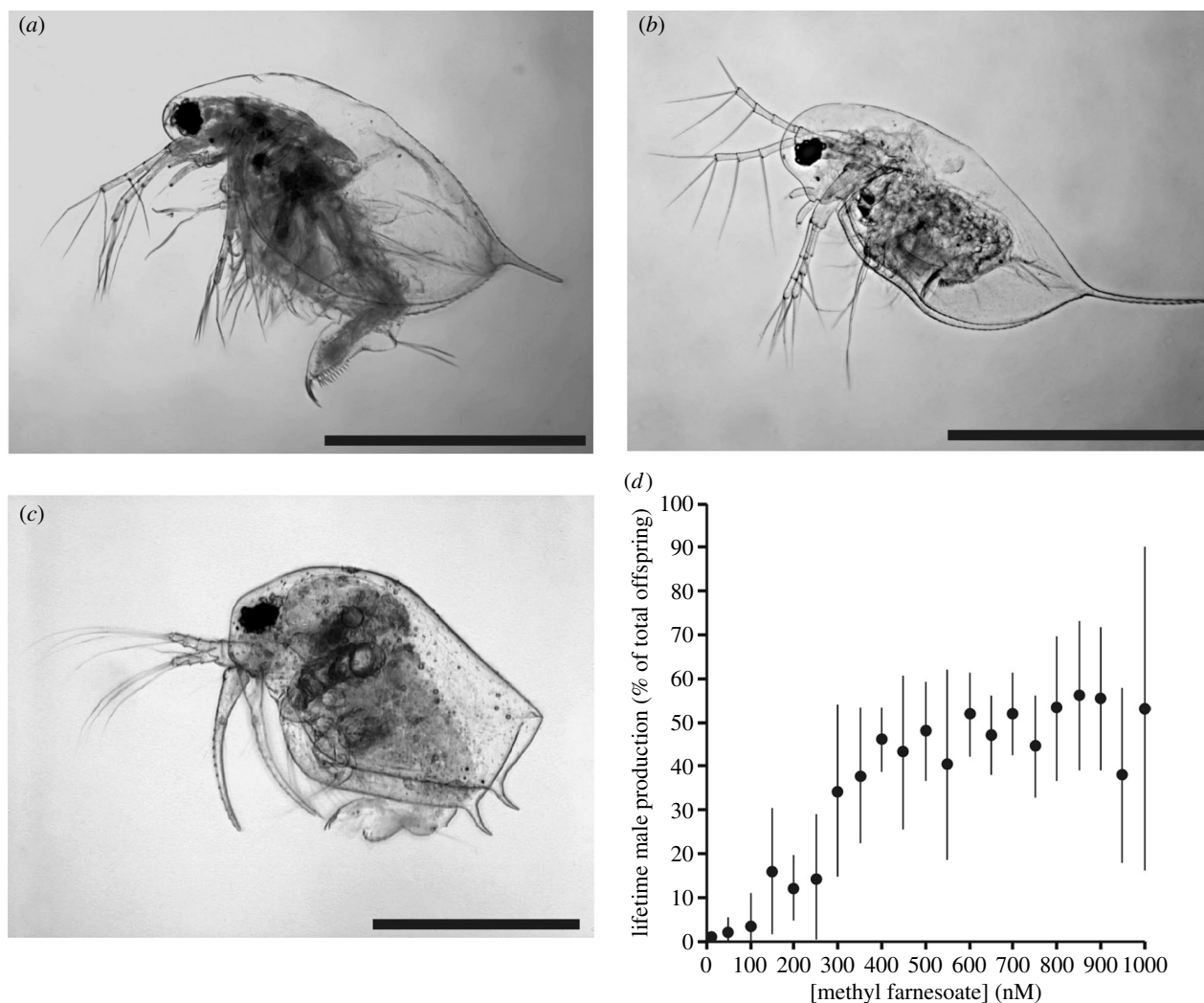


Figure 1. Photographs of induced adult males of Cladocera. (a) *Daphnia pulex*. Scale bar, 1 mm. (b) *D. galeata* × *D. mendotae*. Scale bar, 1 mm. (c) *Bosmina* n. sp. Scale bar, 0.1 mm. (d) Plot of mean lifetime male production as a function of MF concentration in *D. pulex*. Bars represent 95% confidence intervals from five replicates. No males (0%) were observed in the three controls (15 vials) without MF. The [MF] logistic regression curve explained 71% ( $R^2 = 0.71$ ,  $p < 0.0001$ ) of the variance in male production.

## 2. MATERIAL AND METHODS

We collected four lacustrine cladoceran taxa, representing two families and four subgenera. Cultured specimens were identified morphologically and with phylogenetic analysis using mtDNA sequences (ND2 for *Daphnia* and 16S rRNA for bosminids; Taylor *et al.* 2002; Ishida *et al.* in press). *D. pulex* Forbes was collected from Crooked Lake, IN (41°40' N and 85°03' W), *D. galeata* × *D. mendotae* from Lake Erie, Buffalo, NY, *Bosmina* (*Bosmina*) n. sp. from Great Baehre swamp Amherst, NY, and *Bosmina* (*Lunobosmina*) *oriens* De Melo and Hebert from Hell Hollow Pond, CT. The unnamed *Bosmina* species is a member of the subgenus *Bosmina* s. str., and a cryptic sister species to *Bosmina freyi* De Melo and Hebert. *D. pulex* was used for the effect of MF on lifetime male production (the first experiment), because it is amenable to long-term culturing, and the males are well described (Brooks 1953; Alonso 1996). The experimental clone of *D. pulex* was maintained in the lab for more than 2 years at 20 °C temperature and 24 h light photoperiod. *Selenastrum capricornutum* suspensions were used as a food source during culturing and experiments. Under these conditions, the clone of *D. pulex* produced almost exclusively female offspring (more than 99%).

All experiments were carried out under unfavourable conditions for male production: constant daylight (24 h), moderate temperature (20 °C), high food density ( $3.2 \times 10^6$  cells of *S. capricornutum* algae/*Daphnia*/day), and low densities of *Daphnia* (one original female per vial). The culturing solution was made by mixing 10 parts synthetic freshwater (96 mg  $\text{NaHCO}_3$ , 60 mg  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 60 mg  $\text{MgSO}_4$  and 4 mg KCl in 1 l of double distilled water; EPA 2002) with one part algal solution ( $4.8 \times 10^6$  cells  $\text{ml}^{-1}$ ). Each 7 dram borosilicate glass vial (Kimble Glass Inc.) was seeded with a female <24 h old and 20 ml of culturing solution. MF was obtained from Echelon Research Labs (Product number: S-0153). Stock solution was prepared at concentration of 6.6 mM [MF] dissolved in ethanol (up to 0.015% EtOH).

In the first experiment, we set up 21 MF treatments (from 10 to 1000 nM) and three controls: culturing solution, culturing solution with 0.00015% EtOH and culturing solution with 0.015% EtOH. Five replicates were set up for each treatment and control (a total of 120 vials each seeded with single females). All solutions were replaced every 3 days and checked once a day for neonates. Neonates were transferred to vials with culturing solution (without MF) and permitted to grow to adults. Neonate offspring of

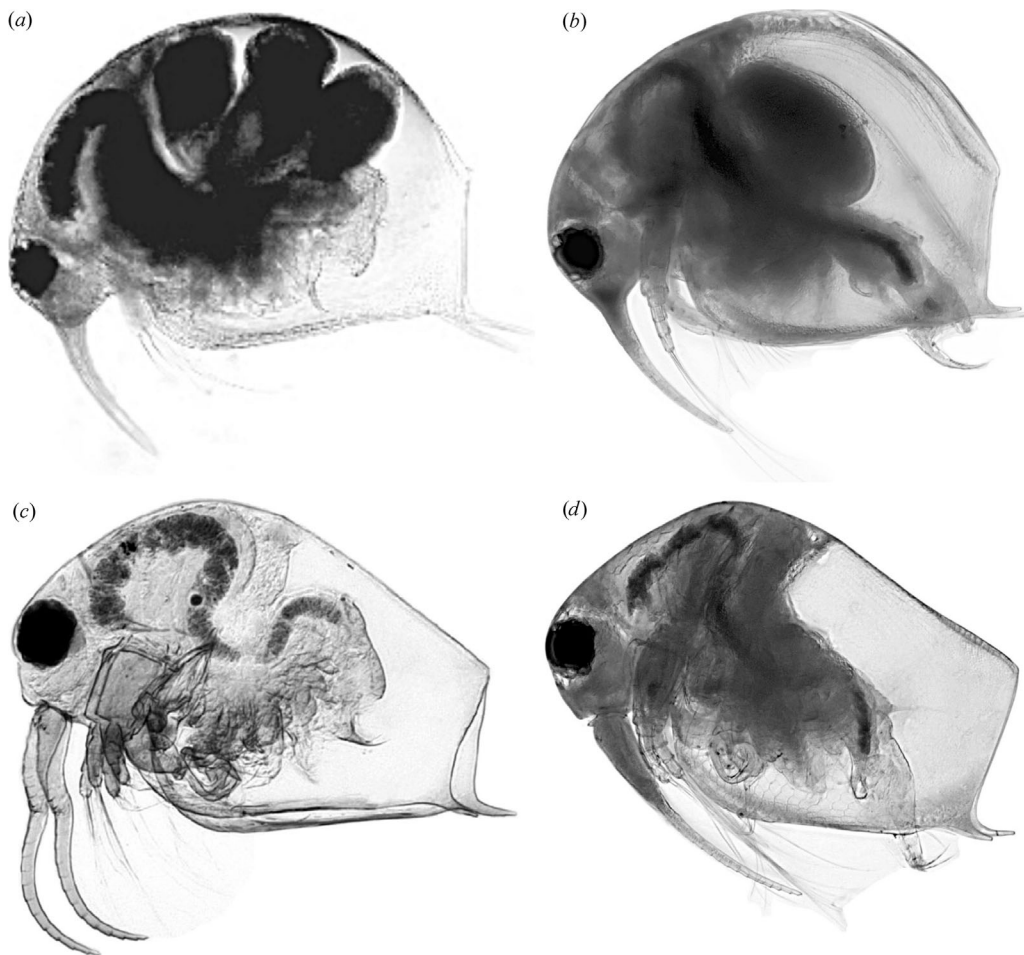


Figure 2. Photographs of cryptic bosminid species showing a greater morphological differentiation among males than among females. All specimens are adults. (a) Female of *Bosmina oriens*. (b) Female of *B. longispina*. (c) Previously unknown MF-induced male of *B. oriens*. (d) Male of *B. longispina*.

*D. pulicaria* were examined for secondary sexual characters under a stereomicroscope, and counted. In *Daphnia*, male traits include movable first antennae, claspers on the first limb, a truncated rostrum and ventral carapace setation (Brooks 1953; Alonso 1996; Dodson & Frey 2001; figure 1a). The experiment continued for the entire lives of the test females (up to three months).

A second, similar set of experiments was set up with the three additional species of cladocerans, but using only an 800 nM MF (the maximum male inducing concentration from the first experiment) treatment and a single control (without MF). Bosminids have similar male secondary sexual characters to *Daphnia*, with moveable antennules, a truncated rostrum and clasping hooks on the first thoracic limbs (Lieder 1983; De Melo & Hebert 1994; figures 1c and 2c). However, the sexual characters are difficult to diagnose in neonate male bosminids. We, therefore, determined the sex of bosminids in mature males.

A multiple comparison analysis, Dunnett's test, was performed to compare each experimental mean with the control mean at the 0.05 significance level. The effect of MF on lifetime male production was tested by logistic regression analysis (SPSS, v. 11.5.0).

### 3. RESULTS

Lifetime male production for *D. pulicaria* increased significantly with MF concentration but levelled at near

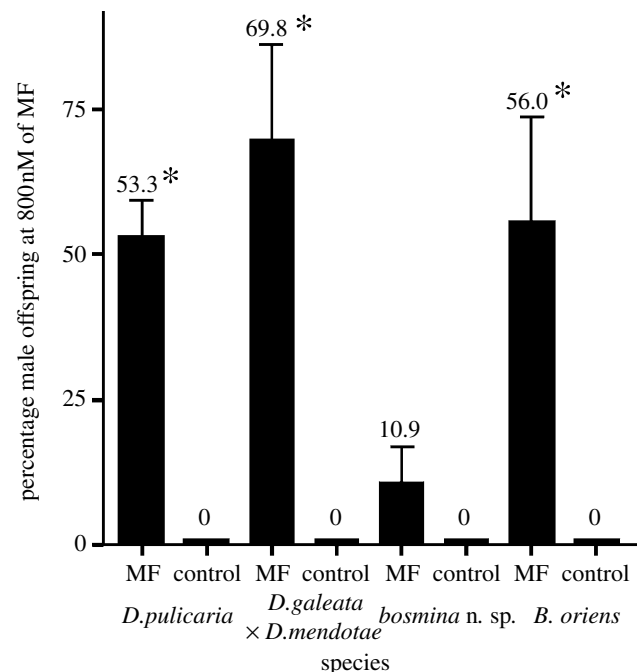


Figure 3. Comparison of lifetime male production (percentage of total offspring) at 800 nM MF of four lacustrine species (*Daphnia pulicaria*, *D. galeata* × *D. mendotae*, *Bosmina* n. sp., and *B. oriens*). Bars show means with 95% confidence intervals. Asterisks indicate that the mean is significantly different from the control (Dunnett's test at  $p < 0.05$ ).

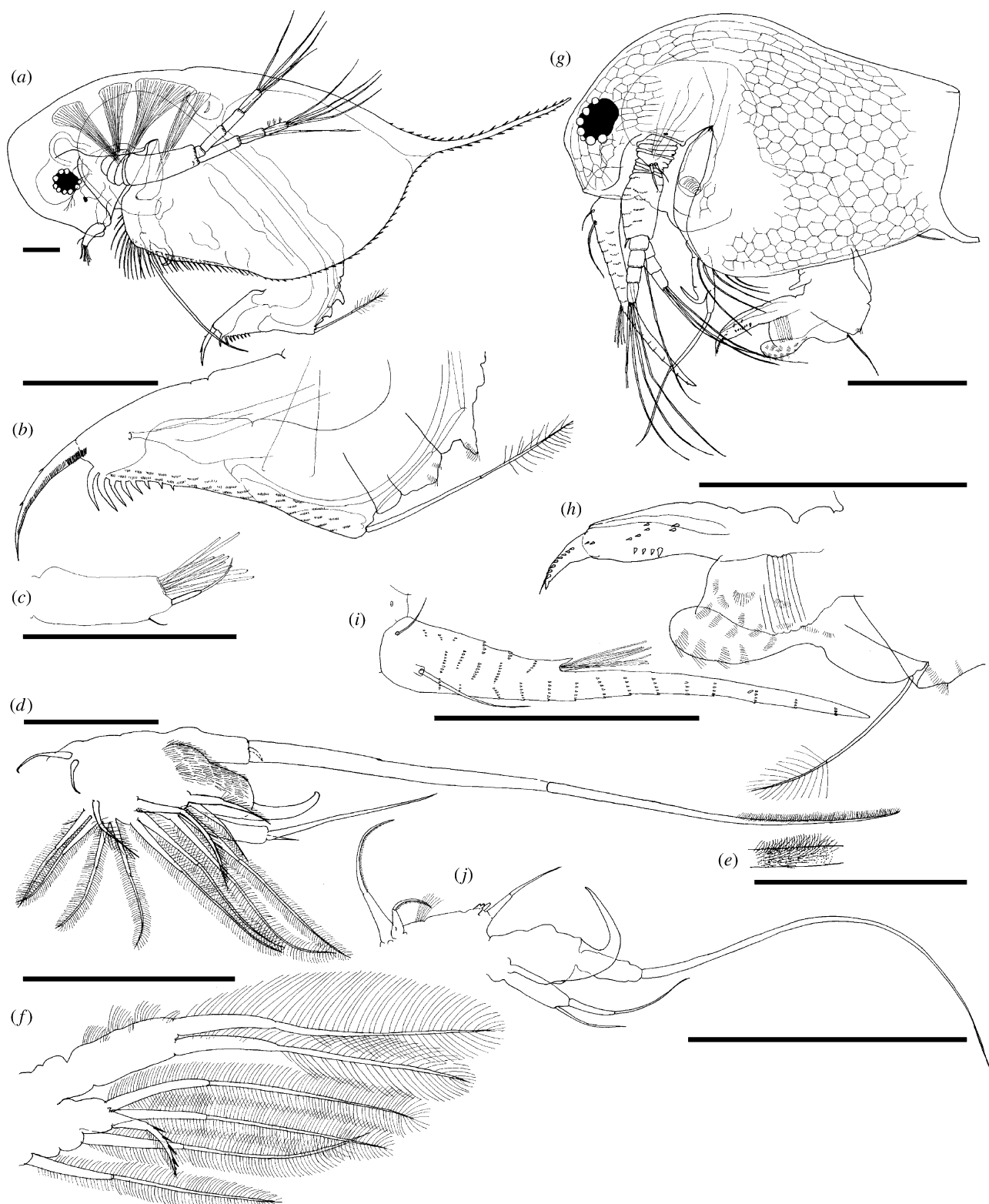


Figure 4. Camera lucida drawings of the induced mature males of *Daphnia galeata* × *D. mendotae* (a–f) and *Bosmina* n. sp. from NY (g–j). Scale bar, 0.1 mm. (a, g) lateral view; (b, h) postabdomen; (c, i) antennule; (d, j) limb I; (e) distal portion of male seta; (f) inner-distal portion of limb II.

50%, when [MF] was greater than 600 nM (figure 1d). Males appeared qualitatively identical in morphology to described, environmentally induced males (Brooks 1953; Alonso 1996). That is, they possessed typical ventral setation, claspers, enlarged first antennae with sensory setae typical for the species. No males were observed in the EtOH or regular controls, indicating that unexpected male-inducing stressors were negligible in our experiments. Unlike other studies (Olmstead & LeBlanc 2000,

2001a, b, 2002; Tatarazako *et al.* 2003; Oda *et al.* 2005), our experiments were carried out under the conditions least favourable for male production (constant light, high food concentration, low density, see Carvalho & Hughes 1983; Korpelainen 1986; Hobæk & Larsson 1990; Kleiven *et al.* 1992). The effect of MF on male induction in *D. pulicaria* was much lower than reported for *D. magna* (at nearly 100% induction for 800 nM MF; Olmstead & LeBlanc 2002).

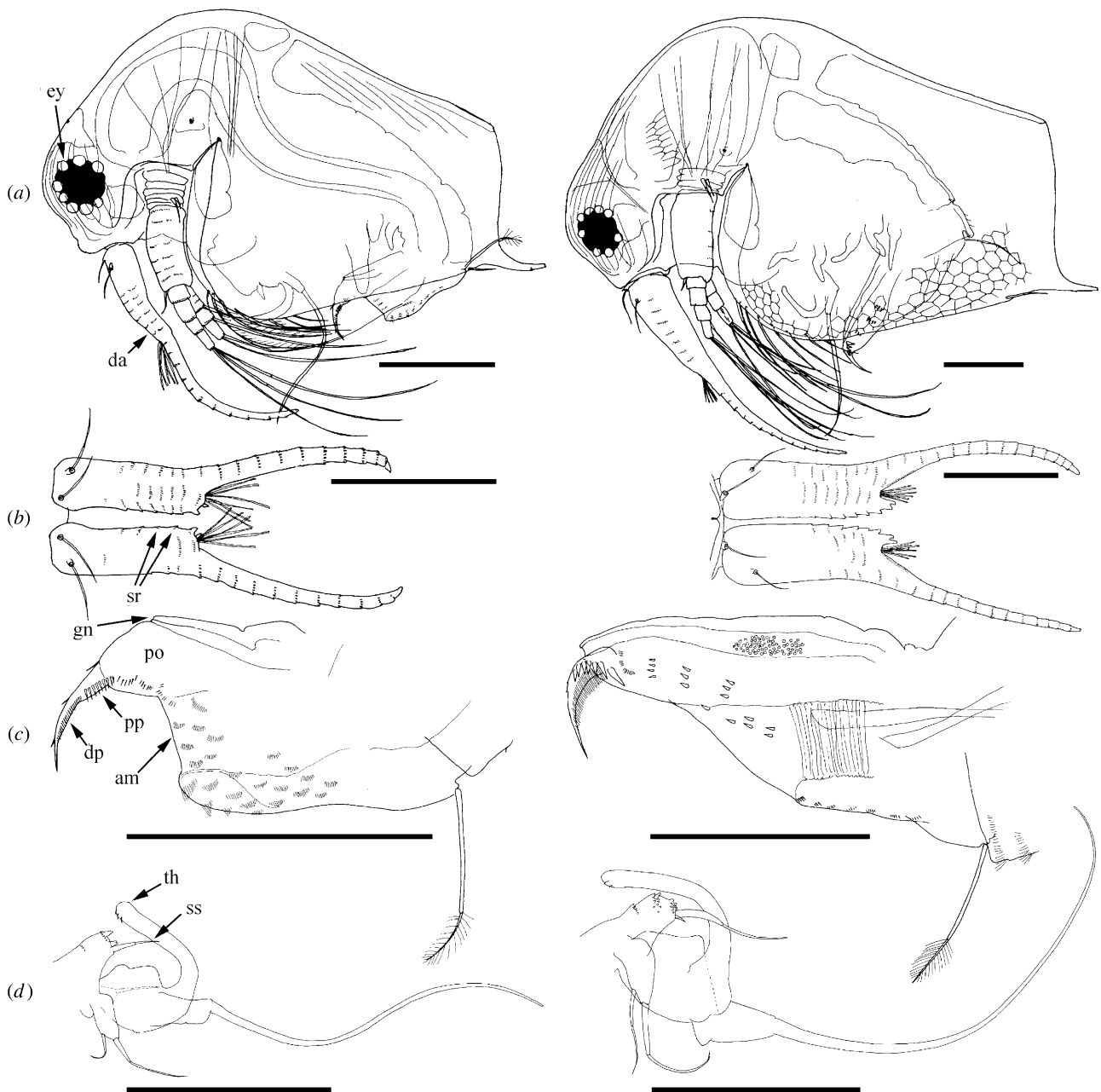


Figure 5. Camera lucida drawings of the induced mature male of *Bosmina oriens* (left column) and the male of *B. longispina* (right column). Scale bar, 0.1 mm. Arrows indicate diagnostic characters that distinguish *B. oriens* from other closely related species such as *B. longispina*. (a) Comparison of male bodies in lateral view showing different shape of antennules. (b) Anterior view of antennules revealing reduced serration in *B. oriens*. (c) Lateral views of postabdomens showing different anal margins, gonopore locations and proximal pecten morphologies. (d) First thoracic limbs with flagellum and hook. am, right-angled anal margin; da, doubly arched antennule; dp, distal pecten on postabdominal claw; ey, compound eye; gn, gonopore; po, postanal portion of postabdomen; pp, proximal pecten on postabdominal claw; sr, serration on inner margin of antennule; ss, seta on subdistal lobe of first limb; th, tip of copulatory hook.

In the second experiment carried out at 800 nM MF, previously unknown males were observed in the MF treatments for each species (figures 1*b,c* and 2*c*), but not in the controls. Male production ranged among species from 10.9% (*Bosmina* n. sp.) to 69.8% (*D. galeata* × *D. mendotae*; figure 3). Again, the males appeared to possess the typical secondary sex features for their respective families.

Here, we briefly describe the previously unknown males. The male of *D. galeata* × *D. mendotae* (figure 4*a-f*) has an elongated body, head with a rudimentary helmet and a slightly depressed ventral margin, ocellus present. Valve with a concave ventral margin armed with setae

decreasing in size posteriorly. A small abdominal projection present only on second (counting from abdomen base) segment. Postabdomen elongated, with concave preanal portion, gonopores open on its sides subdistally. Antennule relatively small, with male seta (flagellum) shorter than longest aesthetasc, sensory seta minute. Limb I with outer distal lobe bearing a rudimentary seta, and a long seta with fully setulated distal portion, inner distal lobe with a small copulatory hook, anterior (stiff) setae on all endites large. Limb II with anterior (stiff) seta on distal endite short and asymmetrically armed, bearing strong denticles along one side and fine setules along other side.

*Bosmina* n. sp. (figure 4g–j) will be formally described in a future article, because at this stage it is difficult to diagnose its morphological differences until detailed description of other species are available. Body relatively high, with somewhat concave dorsal margin of valve. Compound eye large. Distal portion of postabdomen as a tube, with a single gonopore opens distally, preanal margin depressed, with projected preanal angle. Postabdominal claw relatively short, with a sharp terminal spinule. Basal pecten of denticles shifted from postabdominal claw to body of postabdomen, distal pecten consisting of short, robust denticles. Antennule slightly and regularly curved in lateral view, slightly S-shaped in anterior view, serration on its inner margin ill defined. A seta on subdistal lobe of limb I long. Tip of copulatory hook thin, sharp.

*Bosmina* (*Lunobosmina*) *oriens* De Melo and Hebert males are shown in figures 2c and 5 (left column). Body relatively high, with somewhat concave dorsal margin of valve. Compound eye exceptionally large. Antennule double-arched in lateral view, serration on its inner margin ill defined. Postabdomen with truncated, right-angled anal margin and postanal portion un-modified as compared with female, gonopore opens subdistally, at level of anal margin. Postabdominal claw slender, basal pecten of denticles consisting of thin spines, distal pecten consisting of short, fine setules. A seta on subdistal lobe of limb I short. Tip of copulatory hook somewhat inflated.

#### 4. DISCUSSION

The results provide the first evidence that MF can induce male formation in distantly related lacustrine cladocerans. Each of the cladoceran species examined is part of a cryptic species complex, where females of the species are notoriously difficult to tell apart. However, the males of *B. (L.) oriens* showed clear diagnostic morphological differences from the well-known males of the cryptic sympatric species, *B. (Eubosmina) longispina* (figures 2 and 5). Specifically, *B. longispina* males possessed relatively small eyes, evenly curved antennules, well-defined serration on the inner antennule margins, postabdomens with bevelled anal margins and elongated postanal portions, distal gonopore, postabdominal claws with massive spines in the proximal pectens and long setules in distal pecten, long setae on subdistal lobes of limb I, and uninflated tips of the copulatory hooks (figure 5). These morphological differences (apparently of subgeneric rank, see Lieder 1983; De Melo & Hebert 1994; Kotov 1996) support the species designation of *B. oriens* and lend credence to the subgenus *Lunobosmina* that was proposed from DNA sequence analysis (Taylor *et al.* 2002).

We note that such features have been drawn in male bosminids and recorded from paleolimnological sediments in eastern North America (Deevey & Deevey 1971). The existence (albeit rare) of these morphological features in subfossils from lakes from the same region as our source populations make the novel male characters we describe unlikely to be an artefact of MF induction. However, Deevey & Deevey (1971) erroneously assigned these features to *B. longispina*. Instead, males of Holarctic and North American *B. longispina* possess single-arched antennules, massive spines in proximal pecten on postabdominal claw, and a bevelled anal margin (Lieder 1983;

Kotov 1996; figure 5). It is most likely that the subfossils with the doubly arched antennules and right-angled anal margins belong to the previously unknown males of *B. oriens*. We note that our finding of likely diagnostic morphological characters in MF-induced males permits interpretation of older studies and subfossils, where DNA-barcoding comparisons are difficult. The value of hormone-based male induction for other cladoceran species awaits study. Detailed studies of males from several species from the genus *Bosmina* are, for example, necessary to understand the taxonomic significance of male characters in this genus.

An increased understanding of sex determination in cyclic parthenogens offers hope for detailed biological studies in species where males are difficult to induce by standard culturing. However, there is no evidence that MF-induced males are fertile and amenable to breeding studies. Also, MF can cause toxicity to cladoceran embryos and MF can be ineffective in inducing males in obligately asexual females (Mu & LeBlanc 2004; Rider *et al.* 2005). Our evidence from different families suggests that the role of MF in sex determination may be a general phenomenon for cladocerans. Our finding of reduced male induction in lacustrine species (10.9–69.8%) compared to the 100% induction found in two pond-dwelling species (Rider *et al.* 2005) is consistent with a role for MF in the evolution of reduced sexuality in permanent habitats. Nevertheless, the observed differences may be due to the less favourable male-inducing conditions of our experiment, or to inherent differences in the male-induction response of the clones we used (Tessier & Cáceres 2004). Although the existence of a general sex determination role for MF benefits biological studies, insecticides designed to mimic MF may have a more profound effect on freshwater communities than previously considered.

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