

## Production of intersexes and the evolution of androdioecy in the clam shrimp *Eulimnadia texana* (Crustacea, Branchiopoda, Spinicaudata)

STEPHEN C. WEEKS<sup>1\*</sup>, SADIE K. REED<sup>1</sup>, MICHELE CESARI<sup>2</sup> and FRANCA SCANABISSI<sup>2</sup>

<sup>1</sup>*Department of Biology, Program in Evolution, Ecology, and Organismal Biology, ASEC 185, University of Akron, Akron OH 44325-3908, USA*

*Tel. +1 (330) 972-7155; Fax: +1 (330) 972-8445; email: scw@uakron.edu*

<sup>2</sup>*Dipartimento di Biologia Evoluzionistica Sperimentale, Università di Bologna, via Selmi 3, 40126, Bologna, Italy*

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### Summary

The production of low numbers of offspring that exhibit a mixture of male and female traits (termed “intersexes”) is commonly reported for crustaceans. The production of intersexes has been ascribed to both genetic and non-genetic (e.g., parasitic infections and environmental pollutants) causes. Herein we report on two observed types of intersexes in the clam shrimp *Eulimnadia texana*: (1) a “morphological” intersex, possessing secondary male characteristics (e.g., claspers) and an egg-producing gonad, and (2) a “gonadal” intersex, possessing primarily male traits (e.g., male secondary sexual characters and male gamete production) but also producing low levels of abortive female gametes. We propose that these intersexes are likely the products of low frequencies of crossing over between the sex determining chromosomes that result in the array of observed mixed sexual phenotypes. Additionally, we suggest that the low-level production of intersexes, combined with the ephemeral nature of the habitats occupied by these shrimp, may explain the preponderance of androdioecy (mixtures of males and hermaphrodites) found in these clam shrimp, and possibly branchiopods more generally.

**Key words:** Evolution of mating systems, conchostraca, gynandromorphs, hermaphrodites, sex determination, crossing over

### Introduction

Intersexuality (individuals exhibiting a mixture of male and female phenotypes) has been long recognized in a broad array of crustacean taxa (Gissler, 1881; Galil and Tom, 1990; Juchault et al., 1991; Micheli, 1991; Rudolph, 1995; Sassaman and Fugate, 1997; Sillett and Stemberger, 1998; Mitchell, 2001; Barbeau and Grecian, 2003; Hanamura and Ohtsuka, 2003). Such mixed

sex individuals have been termed “gynandromorphs” (Johnson and Otto, 1981; Galil and Tom, 1990; Micheli, 1991; Rudolph, 1995), “pseudohermaphrodites” (Juchault et al., 1991; Moriyasu et al., 1998) and “sexual mosaics” (Sassaman and Fugate, 1997) as well as the more commonly used term “intersex.” In all cases, these intersex individuals appear in low proportion (from <1% up to ~15%) in otherwise dioecious (“bisexual”) species.

\*Corresponding author.

The production of intersexes has been attributed to a variety of sources: parasitic infections (Ginsburger Vogel, 1991), epigenetic cytoplasmic factors (Sassaman and Fugate, 1997), viral infections (Juchault et al., 1991), genetic abnormalities (Parnes et al., 2003), chemical pollutants (Jungmann et al., 2004), hormonal abnormalities (Zou and Fingerman, 2000; Rudolph et al., 2001), and as side effects of environmental sex determination (Dunn et al., 1993; Mitchell, 2001). The various intersexes can either be functional (Dunn et al., 1993; Sillett and Stemberger, 1998; Ladewig et al., 2002; Parnes et al., 2003) or sterile (Sassaman and Fugate, 1997; Barbeau and Grecian, 2003). In functional intersexes, some level of heritability of intersexuality was usually reported (Dunn et al., 1993; Sillett and Stemberger, 1998; Parnes et al., 2003) suggesting some genetic component to the intersexuality in these species.

Although the above variety of mechanisms has been proposed to explain the production of intersexes in crustaceans, including the production of heritable intersexes (Dunn et al., 1993; Sillett and Stemberger, 1998; Parnes et al., 2003), no one has suggested that these intersexes may be produced by low levels of crossing over between the sex chromosomes. Normally, crossing over in the heterogametic sex is selectively disadvantageous specifically because of the possibility of producing intersexes (Bull, 1983). It is thought that this reduction of crossing over eventually leads to the formation of a degenerate sex chromosome due to the inability of the repair of DNA damage in the sex chromosome that is always found in the heterogametic sex [the Y or W (Charlesworth, 2002)]. It is entirely conceivable that in species in which this chromosomal degeneration has not proceeded too far, low levels of crossing over could create intersexes.

There is precedence for suppressed crossing over in crustaceans. Inhibition of crossing over between the sex chromosomes in the heterogametic sex (females) relative to free crossing over in the homogametic sex (males) has been described in the copepod *Tigriopus californicus* (Dill and Burton, 1984). Unlike in other species, where reduced crossing over is limited to the sex chromosomes only (Bull, 1983), crossing over in *T. californicus* is suppressed for all chromosomes in this species. The presumed reason for this limitation in crossing over is to eliminate the creation of dysfunctional intersexes.

In *Eulimnadia texana*, sex determination was initially described as being due to a single sex locus with two alleles (a dominant allele coding for hermaphrodites and a recessive allele coding for males; Sassaman and Weeks, 1993). Since that early work, experimental results suggest that sex is more realistically determined by either a large linkage group or possibly by sex

chromosomes with limited crossing over (Weeks et al., 1999; Weeks et al., 2000; Weeks et al., 2001). Genetic evidence has revealed that three allozyme loci are physically linked to the sex determining region, and that crossing over among these loci is ~1% (Weeks et al., 1999).

Herein we report evidence of intersexes in the androdioecious clam shrimp *Eulimnadia texana* (Packard). We present histological, anatomical and behavioral evidence of mixed sex individuals and offer a unique explanation for their apparent high frequency: intersexes are produced by rare crossing over events between the sex chromosomes in the heterogametic sex. We suggest that this crossing over produces a wide array of mixed sex individuals. In species where “reproductive assurance” (Baker, 1955) might be advantageous, we suggest that the production of intersexes can produce the androdioecious mating systems seen in the genus *Eulimnadia*.

## Methods and Materials

Soil containing clam shrimp eggs was collected from a site in Arizona (previously referred to as the “WAL” site) near Portal in Cochise Co., near the base of the Chiricahua Mountains. These samples were then transported back to the laboratory in Akron, Ohio. Sub-samples of soil (250 ml) from each population were hydrated using deionized water. Hydrations were done in 37-L aquaria under “standard” rearing conditions, which consisted of the following. Aquaria were housed in an environmentally-controlled room under continuous light (Durotest sunlight-simulating fluorescent bulbs), at 25–27°C, and continuous aeration (Sassaman and Weeks, 1993; Weeks et al., 1997). Shrimp were fed 20–40 ml of baker’s yeast solution (1 g dried yeast per 100 ml water) per day per aquarium, depending on the density of shrimp per aquarium.

For the gonadal intersexes, the male shrimps were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.2 M (pH 7.2) for 2 h at 4°C. The specimens were washed in 0.1 M phosphate buffer and then postfixed in 1% OsO<sub>4</sub> in the same buffer for 1 h at 4°C. The samples were processed through a graded acetone series, then propylene oxide and then embedded in Epon-Araldite. The ultrathin sections were observed through a Philips EM 410 electron microscope.

The shrimp described in this study have been culled from a much larger set of clam shrimp. Thus, these examples are not part of a project specifically designed to quantify the abundance of intersexes in this species, but rather are used as examples of the variety of intersexes found in *E. texana*. The “standard” conditions mentioned above should not be considered stressful in

any way, and thus we infer that the production of these intersexes in the laboratory reflect normal processes typical of at least this one population of *E. texana*, and likely the genus *Eulimnadia* generally.

### Results

In this study, we report on two types of intersexes: (1) a “morphological” intersex, and (2) a “gonadal” intersex.

The morphological intersex was randomly found among hundreds of “normal” clam shrimp, and was obvious by the presence of both male secondary sexual characters (e.g., claspers) and the presence of eggs (Fig. 1). This individual had all four male claspers, but was clearly producing eggs. This intersex behaved like a typical male clam shrimp (Medland et al., 2000): it swam faster than the hermaphrodites and was observed clasping onto other hermaphrodites. During clasping, we observed typical thrusting behavior, although we assume that sperm was not successfully transferred due to the production of primarily eggs in the gonad of this animal (Fig. 1).

We isolated this intersex to collect eggs, which were released from the body (signifying a functioning gonopore). However, the animal was unable to hold its eggs in a brood chamber (indicating the lack of a functioning brood chamber). Although the eggs appeared to be developing normally inside the animal (Fig. 1B), when released from the gonopore these eggs were malformed, and did not hatch upon repeated hydrations.

Six males were randomly drawn from a larger set of shrimp and all six were found to be gonadal intersexes. In all respects, these shrimp appeared to be completely male (i.e., all secondary sexual characteristics and behaviors were that of pure males), and thus these males were only determined to be intersexes after their gonads were observed with an electron microscope: early female gonidia in all six observed samples, more advanced stages — to the mature follicle stage — in only two specimens). Thus these individuals were best described as “primarily male” intersexes in which the gonads were made up of somatic cells (the wall) and two types of germ cells. The first and most numerous were male gonidia, very similar to those already described in other branchiopod conchostracans (Wingstrand, 1978). They originated in the wall, intermixed with the monolayered somatic cells, and they completed their maturation inside the gonad lumen. Their prevalence leads us to consider this gonadal type as the true male phenotype. The developmental process of male gonidia in *E. texana* is described in detail in Scanabissi et al. (2006).

The second kind of germ cell is typical of a female gamete. These are present along the wall intermixed

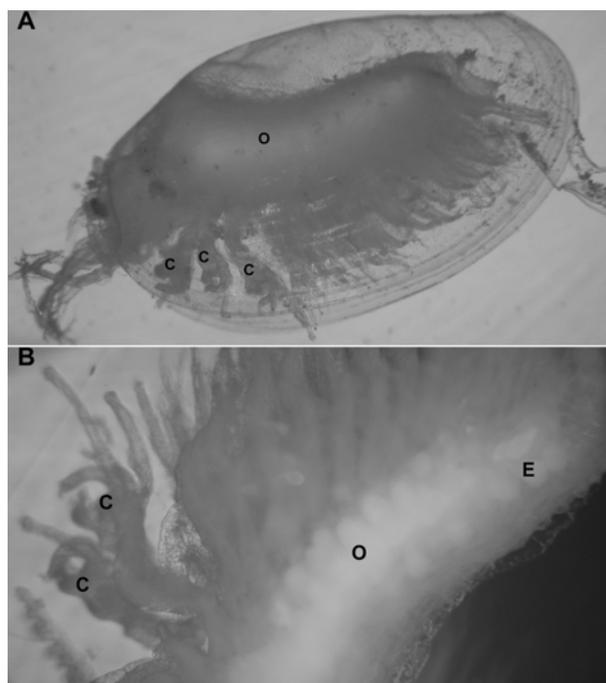


Fig. 1. Morphological intersex, which was a combination of male phenotypes [e.g., claspers (C)] and an ovotestis (O). A. Whole animal picture showing three of the four claspers and the ovotestis. B. Close up of ovotestis showing individual eggs (E) developing in the ovotestis.

with other cells and were found in all males examined, although in much lower numbers than that of the male cells. They were recognizable by a very high nucleoplasmic ratio, a well developed Golgi apparatus, small rounded mitochondria, high cytoplasmic electron density (due to the abundance of ribosomes), and by a stretched cell wall. They are distinguishable from the male germ lineage by their larger size and by the presence of numerous vacuoles, whose diameters remain more or less constant (0.24–0.32  $\mu\text{m}$ ). A ribosome coating is present on the membrane. These vacuoles were never observed in normal male gametes (Fig. 2A).

The development of this “female” gamete then proceeds centripetally towards the gonad lumen, showing the typical maturation of male gametes (a masculinization character in these “female” gametes). This leads to the formation of cells that were larger than typical male gametes (10–15  $\mu\text{m}$ ) with a lower nucleoplasmic ratio, due to enlargement of the cytoplasm and to volume dilatation, resulting in a less electron dense appearance. The nucleus is always well rounded and presents a ring-shaped, condensed chromatin. The most striking features are electron dense globules inside the vacuoles, which give them an endogenous vitellogenetic appearance (Fig. 2B). In fact their production is

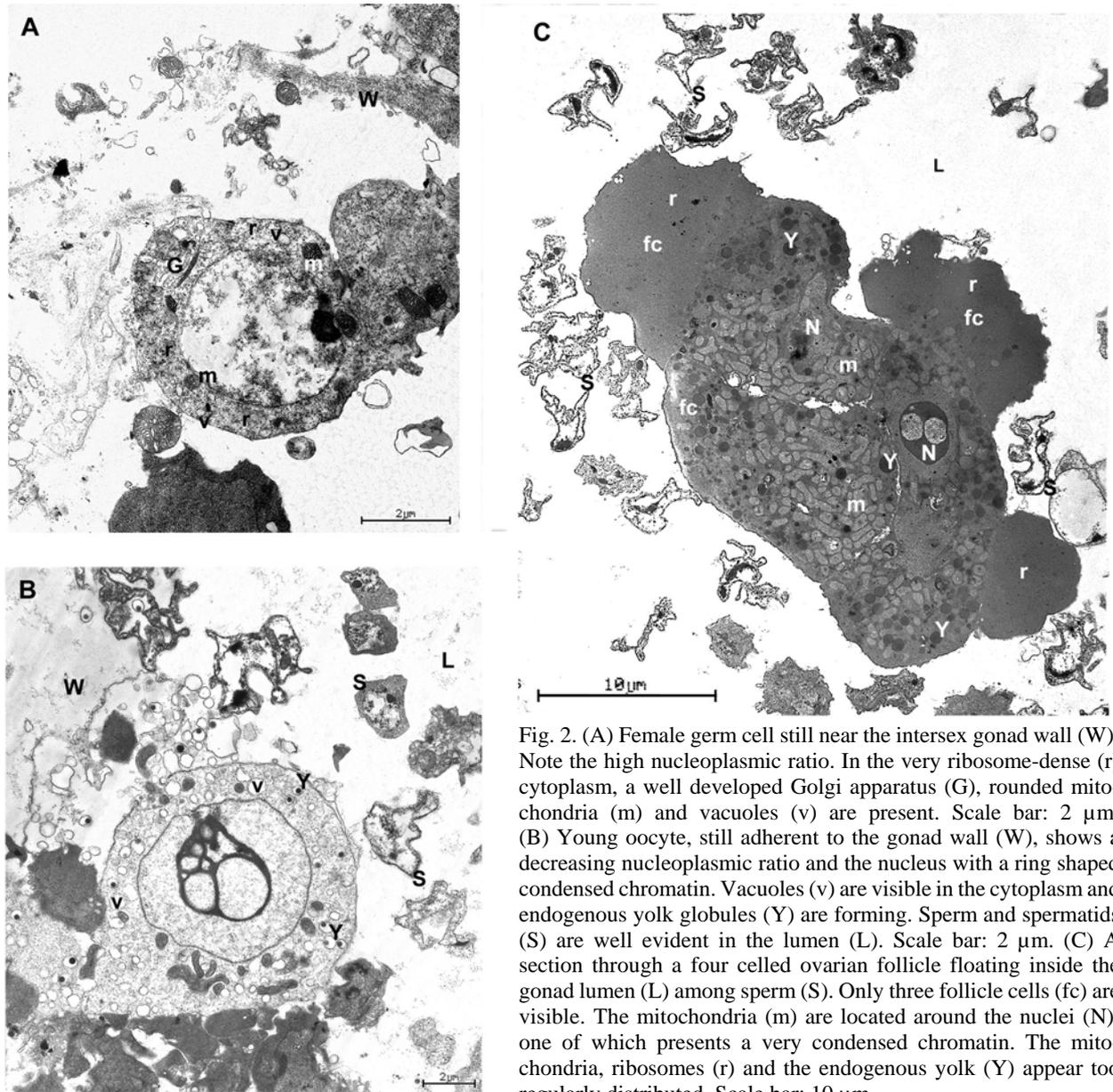


Fig. 2. (A) Female germ cell still near the intersex gonad wall (W). Note the high nucleoplasmic ratio. In the very ribosome-dense (r) cytoplasm, a well developed Golgi apparatus (G), rounded mitochondria (m) and vacuoles (v) are present. Scale bar: 2  $\mu$ m. (B) Young oocyte, still adherent to the gonad wall (W), shows a decreasing nucleoplasmic ratio and the nucleus with a ring shaped condensed chromatin. Vacuoles (v) are visible in the cytoplasm and endogenous yolk globules (Y) are forming. Sperm and spermatids (S) are well evident in the lumen (L). Scale bar: 2  $\mu$ m. (C) A section through a four celled ovarian follicle floating inside the gonad lumen (L) among sperm (S). Only three follicle cells (fc) are visible. The mitochondria (m) are located around the nuclei (N), one of which presents a very condensed chromatin. The mitochondria, ribosomes (r) and the endogenous yolk (Y) appear too regularly distributed. Scale bar: 10  $\mu$ m.

supported by the female germ cells' cytoplasmic organelles. The vitellogenesis always presents two phases in branchiopods, in either Notostraca or Conchostraca: an endogenous and an exogenous phase (Scanabissi Sabelli and Trentini, 1979; Scanabissi Sabelli and Tommasini, 1990; Tommasini and Scanabissi Sabelli, 1992; Scanabissi and Mondini, 2000). The latter involves the extrusion of the ovarian follicle towards the hemocoel, obviously for trophic purposes. However, this has not been ultrastructurally observed in the gonad of the *E. texana* intersexes (Fig. 2B), because maturation of the female gametes continues towards the inside of the gonad lumen, typical of conchostracan male gametes.

The final stage of maturation is represented by a four-celled ovarian follicle, made up of three nurse cells and an oocyte, which are indistinguishable at this early stage. In Fig. 2C, only three out of four cells are visible, due to the angle of sectioning. The follicle is floating among mature sperm in the gonad lumen, and its diameter is 35.5  $\mu$ m. The cytoplasm exhibits extreme ribosome electron density, but only at the cell periphery, while the cell center is filled by numerous mitochondria and a few endogenous yolk globules. The size of follicle cells is very considerable, their diameter ranging from 6 to 24  $\mu$ m, and even the nuclei are up to 11  $\mu$ m in diameter. The chromatin is very condensed but is not

uniform. The follicle is very peculiar because it is naked (i.e., without a follicular cell coating). This is due to maturation inside the gonad lumen versus the maturation towards the hemocoel, which occurs in normally developing eggs dragging the wall cells. In confirmation, no microvilli were observed on the follicle surface, in contrast to what occurs in normal exogenous vitellogenetic oocytes. On the contrary, these intersex cells present aggregations and organelle compartmentalization (i.e., mitochondria and ribosomes), signs of cell affliction, possibly due to an absence of sufficient nutritional resources.

No eggshell-production or glandular type cells were observed along the wall or intermixed with the somatic cells, as one would expect in the female region of a functionally female gonad.

### Discussion

Herein we propose that crossing over between the sex chromosomes (or linked sex determining genes in a larger linkage group) in the heterogametic sex in *E. texana* (termed “amphigenic” hermaphrodites) creates low frequencies of intersexes, such as those reported in this study. Due to the random nature of crossing over, such intersexes would be a somewhat random array of possible male and female phenotypes, spanning the range from primarily male to primarily female, with many other levels of mixed sexual phenotypes in between these two extremes. Herein we report evidence of two sexual mixtures: (1) “primarily male” intersexes, which produce mostly male gametes and abortive female gametes and (2) an intermediate form, producing primarily female gametes (we have no evidence of how many, if any, male gametes were produced by this individual) but having clear male secondary sexual characteristics (e.g., claspers, male mating behavior and lacking a brood chamber). We suggest that the “normal” hermaphrodites in this species are “primarily female” intersexes that have mostly female characteristics (e.g., female mating behavior, presence of a brood chamber, production of mainly female gametes) but have a small section of the gonad producing sperm (Zucker et al., 1997). We propose that these examples are just a few of the many mixtures achievable via random crossing over between the sex chromosomes, and that this type of “evolutionary exploration” of intersex production may have created the original, functional “hermaphrodite” in *Eulimnadia texana* (see below).

The relative abundance of the various intersexes should be determined by the combination of the frequency of their production and by the fitness consequences of the mixed sexual phenotypes they possess. We would expect sterile intersexes, such as the

phenotypic intersex we report herein, to be uncommon due to their immediate natural culling from the population. In fact, we have only found one such intersex in our multiple years of rearing shrimp from this population, which suggests that indeed such sterile intersexes are quite rare. On the other hand, functional intersexes, such as the gonadal intersexes reported herein, could be relatively common. If the production of low levels of abortive eggs in these males does not reduce fertilization success to a large degree, then such intersexes would be nearly neutral recombinants that could drift to relatively high frequencies in smaller, inbred populations. At the opposite end of the spectrum, intersexes that confer a selective benefit (e.g., by allowing “reproductive assurance”) could easily spread to fixation in such species (see below).

Assuming that crossing over between the sex chromosomes creates the observed intersexes (i.e., instead of some non-genetic mechanism), *E. texana* may “sample” a variety of intersex types over generations. Such sampling may have allowed the creation of the first functional “hermaphrodite” in this species, which was likely derived from a dioecious ancestor (Sassaman, 1995; Hoeh et al., 2006). Because such crossing over produces a heritable intersex that can be passed down through generations, once an initially viable intersex was produced (theoretically one that was primarily female but which had a small amount of the gonad allocated to sperm production; Pannell, 2002), further “refinement” of the intersex, via additional crossing over events or supporting mutations, could occur to produce a viable hermaphrodite.

If our notion of intersex creation in *E. texana* is correct, we may also be able to explain the abundance of androdioecy in branchiopods more generally. Androdioecy has been described in thirteen species of *Eulimnadia* (Weeks et al., 2006) and in *Triops newberryi* (Sassaman, 1991). The preponderance of intersexes in the branchiopods (Sassaman and Fugate, 1997) combined with their tendency to occupy ephemeral habitats may make this group predisposed to the evolution of androdioecy. The estimate of 1–2% of offspring from heterogametic, branchiopod parents being products of crossing over of the sex chromosomes (Weeks et al., 1999) would allow a constant influx of a low level of “pseudo-hermaphrodites.” If hermaphroditism would benefit branchiopod species in temporary pools, then we might expect a number of cases in which functional intersexes were selected in this group, thus creating mixtures of males, females, and the newly created “hermaphrodites.” Such a mixture of males, hermaphrodites and females has been termed “trioecy,” which has been suggested to be highly unstable, and usually breaks down to either androdioecy or gynodioecy

(mixtures of females and hermaphrodites; Wolf and Takebayashi, 2004). In branchiopods, which have life histories similar to those envisioned by Pannell (2002), we may expect that androdioecy is the most likely outcome. This may explain the observation of a higher rate of androdioecy in the branchiopods than in any other animal taxon (Weeks et al., 2006).

In conclusion, we suggest that the finding of a low number of mixed sex (i.e., “intersex”) individuals in *E. texana* and other crustaceans may be the product of limited crossing over between the sex chromosomes in the heterogametic sex of these species. In most cases, such production of intersexes is likely selected against (Bull, 1983), but for species regularly occupying habitats with low population sizes, intersexes that can gain “reproductive assurance” via self fertilization may be selectively advantageous (Pannell, 2002). We suggest that this may be the case in many branchiopod species, which could explain the preponderance of branchiopods with the otherwise rare mating system of androdioecy. We hope that other crustacean researchers will be able to test whether crossing over explains the creation of intersexes in other crustacean species, while we further explore this idea in the genus *Eulimnadia*.

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