Determining Sexual Dimorphism of Living Aquatic Beetles, *Stygoparnus comalensis* (Coleoptera: Dryopidae) and *Heterelmis comalensis* (Coleoptera: Elmidae), Using Internal Abdominal Structures

Ely Kosnicki

BIO-WEST, Inc., 1405 United Drive, Suite 111, San Marcos, TX 78666 (ekosnicki@bio-west.com)

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Abstract

The Comal Springs dryopid beetle, *Stygoparnus comalensis* Barr and Spangler, and the Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, and Brown, are protected by the United States Fish and Wildlife Service (USFWS), and the development of a captive self-propagating refuge is of importance to stakeholders within the Edwards Aquifer. Being able to reliably distinguish the sex of living subjects is desirable for establishing a successful refuge program. Ventrite, elytron, and pronotum measurements of *S. comalensis* were taken to determine if there were sexually dimorphic allometries. Various lighting techniques were also implemented to see if there were other characters that could potentially be used to distinguish females and males. Measurements were not found to satisfactorily separate sexes; however, lateral lighting was found to consistently illuminate internal abdominal structures of *S. comalensis* where sternite 8 was viewable in males and the fused gonocoxites were viewable in females. Lateral lighting was used to examine living specimens of *H. comalensis*, and it was found that sternite 8 could be viewed in both sexes where the anterior strut of females was much longer and discernible from the anterior strut of males. Commentary regarding the use of cameras and photography for observing living subjects is given.

Key words: sexual dimorphism, lateral lighting, dryopid beetle, riffle beetle, subterranean

The Comal Springs dryopid beetle, *Stygoparnus comalensis* Barr and Spangler, is a troglobitic beetle in the family Dryopidae (Coleoptera) known primarily from Comal Springs, Comal County, TX, but has also been collected from several springs in Hays County, TX (Gibson et al. 2008). The Comal Springs riffle beetle, *Heterelmis comalensis* Bosse, Tuff, and Brown, is an aquatic beetle in the family Elmidae (Coleoptera) known primarily from Comal Springs, Comal County, TX, but has also been collected from San Marcos Springs, in Hays County, TX (Gibson et al. 2008). Both species are protected by the United States Fish and Wildlife Service (USFWS 1997). Twenty-two and 15.56 ha of surface critical habitat have been designated for *H. comalensis* and *S. comalensis*, respectively, at Comal Springs, San Marcos Springs, and Fern Bank Springs (USFWS 2013). All localities are part of the Edwards Aquifer, one of the most productive aquifers in the United States with a surface area of ca. 9,325 km² located in central Texas; the aquifer is the primary source of water for the city of San Antonio, other surrounding municipalities, and agricultural production (Edwards Aquifer Authority 2019. https://www.edwardsaquifer.org/science-and-maps/about-the-edwards-aquifer, accessed 13 June 2019).

Like many species of the Edwards Aquifer, *S. comalensis* and *H. comalensis* face numerous threats to their ecosystem, especially, overpumping of water, pollution, and competition from introduced species (Bowles and Arsuffi 1993). Having functional refuges that contain self-propagating captive populations is a goal of the Edwards Aquifer Habitat Conservation Plan (EAHCP). An understanding of how to distinguish sexes in living individuals is essential for performing mating experiments for the purpose of improving husbandry practices toward meeting that goal.

The determination of sex for members of the families Dryopidae and Elmidae typically requires dissection to expose the genitalia; however, this procedure is lethal to living subjects. A patient observer may occasionally catch a glimpse of the end of an everted ovipositor or penis while examining a living beetle with a stereoscope; however, it is unlikely that most individuals will be cooperative.

The description of *S. comalensis* included measurements that showed a substantial overlap between sexes, with males being slightly larger (Barr and Spangler 1992). It was also shown that males had tufts of fine, sparse, longer setae on their metasternum, while females did not. However, these tufts are very difficult to see in
preserved specimens, requiring specific lighting and positioning (Fig. 1). Using this character to separate living individuals is impractical because increased handling risks damage to the subject, and a poor viewing could result in a misidentification of a male for a female.

The description of *H. comalensis* states that the female is slightly larger than the male, but no other externally visible features have been noted to distinguish the sexes (Bosse et al. 1988). More recently, length measurements were used to distinguish sexes (BIO-WEST riffle beetle life history study, final report, 2017); however, there was an overlap in sizes, rendering uncertainty for a number of individuals at an error rate of ca. 10%, based on the length of ventrite 5.

Some species of dryopids display external features that are sexually dimorphic. Males of *Helichus fastigatus* (Say) have tubercles on their hind coxae (Young 1954). Darlington (1936) indicated that he could separate sexes of several species of *Phanocerus* Sharp by the shape of the body. Males of the dryopid species *Geoparmus loebli* Kodada, Kaducb and Šiampor have small projections on their meso- and metatibia (Kodada et al. 2013).

Similarly, morphologies have been distinguished in some elmid species as well. Males of some species of *Stenelmis* Dufour have tibial ridges (Epler 2010). Males of a number of species of *Graphelmis* Delève are distinguished from conspecific females by the presence of differentiated setae on the disc of the metasternum with or without intercoxal processes, upturned anterior labral margins, and/or the presence of metatibial spines, serrations, or outgrowths (Čiampor 2001, 2002, 2003, 2004). Males of *Pseudamophilus davidi* Kodada have an emarginate apex of ventrite 5, while females do not. Females of several species of *Vietelmis* Delève have a more rounded ventrite 5, lack tooth-like projections, and lack peg-like seta or other projections on the metatibia (Kodada and Čiampor 2001, 2002, 2003, 2004). Males of *Stygoparnus comalensis* have a distinctive distal process of ventrite 3 (Lašsová et al. 2014). Less often, externally viewable internal characters have been used to distinguish sexes of living elmids (Fernandes et al. 2010).

The objective of this study was to identify sexually dimorphic characters that could be used reliably to initiate mating pairs for fecundity studies of *S. comalensis*. After characters for *S. comalensis* were identified, similar characters were examined on living subjects of *H. comalensis* to see if they could be used to reliably separate sexes.

**Materials and Methods**

At the time this study was developed, *S. comalensis* was considered rare and difficult to collect; at the beginning of 2018 the USFWS only had 15 living adults in refuge (Edwards Aquifer Habitat Conservation Plan, annual report, 2017). Nine female and six male preserved *S. comalensis* adult specimens were examined from a collection at the USFWS San Marcos Aquatic Resources Center (SMARC). Specimens were submerged in a watch glass of 70% isopropyl alcohol and positioned underneath a coverslip for dorsal and ventral views. After positioning, excess isopropyl alcohol was pipetted out to compress the specimen underneath the coverslip. Photographs were taken with a NIS-Elements imaging source package, including acquisition and analysis software, and a HD color camera (Nikon Corporation, Tokyo, Japan). The camera was mounted on a Nikon SMZ 18 stereo scope, and measurements were later taken with the cellSens standard imaging software version 1.18 (Olympus Corporation, Tokyo, Japan). Length and width measurements were taken of each ventrite along with the lengths of the pronotum and elytra, respectively. These characters were selected because they were relatively easy to photograph and little manipulation would be required to examine these features when working with living subjects. After specimens were photographed, their genitalia were dissected to determine their sex.

Stepwise analysis (alpha to enter or to remove = 0.20) was used as a first step to select useful characters for developing a multivariate model for differentiating between the sexes. Considering only one variable was found to be useful (see Results below), percentiles were used to find size ranges for each sex based on that character. Validation was performed by applying these ranges to 20 living individuals obtained from Comal Springs under permit from state and Federal authorities. Living beetles were placed on a watch glass with a plastic pipette. A coverslip was placed over the subject, and excess water was pipetted out to restrain the insect while still keeping it submerged underneath the coverslip (no air bubbles). After photographing, water was added back to the watch glass to free the individual and remove the coverslip. The subject was then pipetted back to its holding container. Measurements were later taken from the photographs as described above. Stepwise analysis was conducted on the suite of measurements (see Table 1) with the greedy.wilks function of the klaR and MASS packages for R statistical software version 3.4.1 (R Core Team 2017).

During the process of photographing preserved specimens and interacting with living individuals of *S. comalensis*, other features were inspected. Different lighting strategies consisting of overhead, back, and lateral lighting and combinations of overhead with back lighting and lateral with back lighting were employed to find the optimal illumination for viewing various characters. The lateral lighting technique was employed by directing the fiber optic lights at the subject, opposite of each other, through the sides of the watch glass (Fig. 2). After a lighting technique was consistently found to view primary and secondary sex characters of *S. comalensis*, the same lighting technique was applied.

![Fig. 1. Long setal tufts of *Stygoparnus comalensis* found on the metasternum of males. Arrows indicate the tufts.](image)

**Table 1.** Means and 1 SD of 11 measurements of nine female and six male *Stygoparnus comalensis* specimens

| Measure          | Female           | Male            |
|------------------|------------------|-----------------|
| Length ventrite 1| 0.42 ± 0.03      | 0.39 ± 0.02     |
| Length ventrite 2| 0.31 ± 0.02      | 0.28 ± 0.03     |
| Length ventrite 3| 0.24 ± 0.03      | 0.21 ± 0.03     |
| Length ventrite 4| 0.22 ± 0.03      | 0.17 ± 0.03     |
| Length ventrite 5| 0.43 ± 0.04      | 0.43 ± 0.02     |
| Width ventrite 2  | 1.02 ± 0.07      | 0.99 ± 0.05     |
| Width ventrite 3  | 0.97 ± 0.07      | 0.96 ± 0.04     |
| Width ventrite 4  | 0.84 ± 0.07      | 0.83 ± 0.04     |
| Width ventrite 5  | 0.67 ± 0.04      | 0.62 ± 0.03     |
| Elytron length    | 2.15 ± 0.10      | 1.98 ± 0.08     |
| Pronotum length   | 0.88 ± 0.05      | 0.83 ± 0.03     |
to living subjects of *H. comalensis* obtained from the SMARC refuge. Slides of internal structures that were repeatably visible in undissected specimens of both species were prepared by clearing in KOH 10% solution overnight, dehydrating with 200 proof EtOH, and mounting with euparal. Photographs of undissected, partially dissected, and cleared dissections of internal structures were sent to specialists for verification of the body parts. Measurements for *H. comalensis* were not included in this study since they have already been conducted (BIO-WEST riffle beetle life history study, final report, 2017).

**Results**

Measurement results of characters of the preserved specimens are presented in Table 1. The elytron length was the only measurement to show potential for distinguishing between sexes of *S. comalensis* with a correctness rate of 0.8 ($F$-value = 11.79; $P$-value = 0.004). Using the 10th and 90th percentiles for females and males, respectively, individuals with an elytron length > 2.06 mm were considered females; otherwise they were considered males. From this partition, living females were misidentified six out of 11 times (55%) and males three out of nine times (33%). Combining the 15 specimens and 20 living subjects with measured elytra (20 females; 15 males), the percentiles shifted to the 30th and 70th, representing females > 2.00 mm and males < 1.98 mm, respectively, at an error rate of 30%. Elytral sizes ranged from 1.86 to 2.19 mm in males and 1.82 to 2.35 mm in females.

When preserved specimens and living subjects were placed on their dorsum and a lateral lighting technique was employed (Fig. 2),
internal structures of *S. comalensis* could be viewed (Fig. 3). There was a strong contrast of ventrite 5 between females (Fig. 3a) and males (Fig. 3e). The sclerotized anterior end of abdominal sternite 8 was discernible through ventrite 5 in males (Fig. 3e); this structure was confirmed by dissection (Fig. 3f) and clearing (Fig. 3g). Conversely, abdominal sternite 8 appeared light or unperceivable in females (Fig. 3a, top arrow); however, the fused gonocoxites could readily be seen through ventrite 5 in females (Fig. 3a, bottom arrow); these structures were confirmed through dissection (Fig. 3b and c) and clearing (Fig. 3d). The same lighting technique was applied to living individuals of *H. comalensis* obtained from the SMARC refuge. Similarly, there was a strong contrast between ventrites 2–5 of the female (Fig. 4a) and the male (Fig. 4d). The female sternite 8 was highly visible with the anterior strut extending to ventrite 2 (Fig. 4a); this structure was confirmed by dissection (Fig. 4b) and clearing (Fig. 4c). Sternite 8 of the male elmid (Fig. 4d) was similar to the dryopid (Fig. 3e), with a short anterior strut; this structure was confirmed by dissection (Fig. 4e) and clearing (Fig. 4f). Furthermore, the female strut of *H. comalensis* was thicker than that of the male, with the lateral margins heavily sclerotized and appearing to be two separate bars converging through ventrite 4 or 5 (Fig. 4a), while the male strut was found to be thinner, appearing as a single bar (Fig. 4d). These characters were best viewed when fiber optic lights were positioned lateral to the watch glass with no back lighting.

**Discussion**

The ability to quickly identify the sex of an individual without having to restrain it should be highly attractive when working with endangered beetles. Restraining with a coverslip to take a photograph for measurement causes noticeable trauma to subjects and likely increases the risk of mortality. The lateral lighting technique does require placing an individual on its back (with or without a coverslip), but the trained eye can identify the sex of both species within seconds. Throughout this study, it was rare to encounter an adult *S. comalensis* or *H. comalensis* that was not readily identifiable as a female or male using the characters and lighting technique described above. However, on one occasion, a female *S. comalensis* could not be sexed because

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**Fig. 4.** Internal structures of *Heterelmis comalensis* adults used for determining sex; (a) lateral lighting view of a female with the upper arrow pointing to the anterior strut of sternite 8 and lower arrow pointing to sternite 8; (b) partially dissected female sternites with upper arrow pointing to the anterior strut of sternite 8 and the lower arrow pointing to sternite 8; (c) dissected and cleared female sternal segments with the upper arrow pointing to the anterior strut of sternite 8, the middle arrow pointing to the right gonocoxite, and the lower arrow pointing to sternite 8; (d) lateral lighting view of a male with the arrow pointing to sternite 8; (e) partially dissected male sternites with the upper arrow pointing to the anterior strut of sternite 8 and the lower arrow pointing to sternite 8; (f) dissected and cleared male sternal segments with upper arrow pointing to the anterior strut of sternite 8 and the lower arrow pointing to sternite 8, the aedeagus is shown in relation to sternite 8.
its gonocoxites were missing; evidence of damage was apparent in the specimen’s abdomen after postmortem dissection. It is also interesting to note that the metasternal setal tufts on male S. comalensis were only viewable with a stereoscope by the lateral lighting technique. This lighting technique is not a unique method; it is used by taxonomists and production taxonomists (e.g., Stribling et al. 2003) because alternative lighting schemes illuminate different structures.

The anterior sternite 8 in elmid species is often sexually dimorphic, with the female sternite being much longer than the male (e.g., Ciampor 2001, Yoshitomi and Jeng 2013), and has been used to delineate sex without dissection in other elmid species (Fernandes et al. 2010). However, in some species these structures appear to be reduced (Kodada 1992), obliterated (Laššová et al. 2014) or well developed and subequal in length between sexes (Kodada and Ciampor 2000). Sternite 8 shows similar sexual dimorphic characters in dryopids as well (Kodada et al. 2009, 2013), though these structures are often lacking in species descriptions.

In general, measurements of segments and body parts were poor for distinguishing between sexes of S. comalensis. This may be due, at least in part, to the low sample size of specimens examined. However, larger female sizes in this study were not supported by the original description; Barr and Spangler (1992) found males to be slightly, though not significantly, larger than females with elytral length ranges of 2.08–2.40 mm and 2.00–2.40 mm, respectively, which were also slightly longer than the elytra lengths measured in this paper. It should be noted that adult size may vary due to environmental conditions. Size differences from the original description may be attributed to the fact that this study took measurements using computer software; however, it cannot be ruled out that individuals may be smaller than historic records due to unidentified factors. Furthermore, different populations may have slight differences in size ranges, which would require a sexing technique with separate sets of measurements for each population.

Photography is an important tool that helps to convey information, and certainly this study benefited by using the technology to help verify structures. However, photographs are one step removed from looking at a specimen through the stereoscope; resolution is diminished, and one is not able to effectively manipulate subtle angles that give the viewer an enhanced perspective of characters she/he is inspecting. As imaging systems are now becoming standard equipment in laboratories, there seems to be a shift to looking at specimens on a monitor rather than looking through eyepieces. Many fine details are lost on screen. Furthermore, it is more difficult to observe live images on a screen, and so some people tend to take a photograph and look at that rather than the actual subject. Observing living insects directly through eyepieces of a stereoscope gives one a much better appreciation for the subtle movements and behaviors of the study organism.

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