

Helobdella blinni sp. n. (Hirudinida, Glossiphoniidae) a new species inhabiting Montezuma Well, Arizona, USA

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Abstract

A new leech species *Helobdella blinni* sp. n., is described from Montezuma Well, an isolated travertine spring mound located in central Arizona, USA. In its native habitat, *H. blinni* had been previously identified as *Helobdella stagnalis* (Linnaeus, 1758), which was later reclassified to *Helobdella modesta* (Verrill, 1872). Similar to the European *H. stagnalis* and North American *H. modesta*, *H. blinni* has six pairs of testisacs, five pairs of smooth crop caecae, one lobed pair of posteriorly-directed crop caecae, one pair of eyes, a nuchal scute, and diffuse salivary glands. However, the pigmentation of this new species ranges from light to dark brown, unlike *H. modesta* which tends to be light grey in color. Also, *H. modesta* produces a clutch of 12–35 pink eggs, whereas *H. blinni* produces smaller clutches of white eggs (7–14, 0.5 ± 0.15 mm, N = 7) and consequently broods fewer young (1–14, 7 ± 3.3 mm, N = 97). *Helobdella blinni* are also able to breed year-round due to the constant warm water conditions in Montezuma Well. Their breeding season is not restricted by seasonal temperatures. These species are morphologically similar, however, comparing the COI mtDNA sequences of *H. blinni* with sequences from nearby populations of *H. modesta* and other *Helobdella* species from GenBank indicate that *H. blinni* is genetically distinct from these other *Helobdella* populations.

Keywords

Leech, Glossiphoniidae, *Helobdella blinni* sp. n., new species, Montezuma Well

Introduction

Montezuma Well is a collapsed travertine spring mound located 72 km south of Flagstaff in the Verde Valley of Northern Arizona (34.6491°N, 111.7522°W (DD)) (Fig. 1A). The age of Montezuma Well is estimated to be ~11,000 years (Wagner and Blinn 1987). This location is thermally constant year-round (19–24°C) and is continuously replenished by two vents located at the well bottom. Montezuma Well is 0.76 ha in area and approximately 20 m deep. Most of the shoreline drops off immediately into open water, except at the northeast corner where water drains through a shallow region called the “swallet” and empties into Wet Beaver Creek which is located east of Montezuma Well (Fig. 1A–B). The water within Montezuma Well has unique water chemistry, containing high levels of arsenic (>100µg/L) and dissolved CO₂ (>300mg/L) (Cole and Barry 1973).

Four leech species are known to inhabit Montezuma Well, including an endemic pelagic predator (Govedich et al. 1998), the erpobdellid *Motobdella montezuma* (Davies et al. 1985), and three other glossiphoniid species currently identified as *Helobdella papillata* (Moore, 1952), *H. elongata* (Castle, 1900), and a species currently thought to be *H. stagnalis* (Linnaeus, 1758), all of which inhabit the swallet (Fig. 1B). These Montezuma Well leech populations are thought to have been isolated from other leech populations for as long as 11,000 years (Wagner and Blinn 1987).

In support of this hypothesis, Beresic-Perrins’s (2010) description of brood size, parental behavior, and life history of the Montezuma Well population of *H. stagnalis* suggests that this leech is distinct from other known populations of *H. stagnalis*, a species originally described from Europe and which had until very recently been considered to be a widespread cosmopolitan leech species, inhabiting both Europe and North America. Siddall et al. (2005) addressed this problem by resurrecting the original species description for the North American leech, *Helobdella modesta* (Verrill, 1872) which had long been considered to be a synonym of the European *H. stagnalis* (Moore 1898). The molecular analysis by Moser et al. (2011) provided confirmation for the resurrection of *H. modesta* by Siddall et al. (2005). Even though the two species are morphologically indistinguishable (Verrill 1872, Moore 1898, Moore 1952), they differ genetically. Henceforth, we will refer to the North American *H. stagnalis* as *H. modesta*.

Here, we compare key traits, both morphological and molecular, among members of the Montezuma Well *Helobdella* sp. population, several other nearby populations of *H. modesta*, and several other *Helobdella* species. Our molecular analysis includes the cytochrome *c* oxidase subunit I (COI) mitochondrial gene region to test the hypothesis that the Montezuma Well population of *H. modesta* is a distinct species and warrants a new species description. This region is known to be sufficiently variable to reveal interspecific differences and unlikely to suggest differences due to elevated mutation rates (Apakupakul et al. 1999).

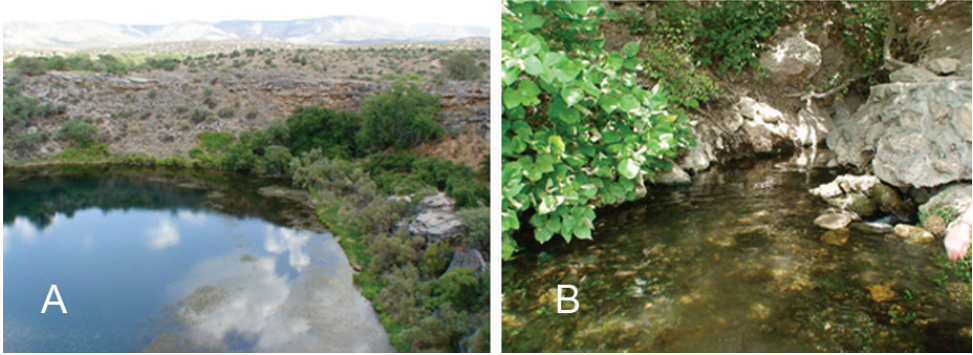


Figure 1. Location of *Helobdella blinni* sp. n. **A** The northeast side of Montezuma Well; and **B** The swallet where the leeches were collected.

Materials and methods

Sampling

A total of 34 individuals of *Helobdella* sp. inhabiting Montezuma Well were collected from the underside of rocks in the swallet: five specimens were collected in June 2011 for molecular analysis and 29 were collected in June 2012 to assess morphological characteristics. For the molecular analysis, the leeches were preserved in 95% ethanol and others, for museum collections, were fixed with buffered formalin overnight and preserved in 70% ethanol. Additionally, a total of 10 specimens of *H. c.f. modesta* from Rio de Flag ponds near the Rio de Flag Waste Water Facility outflow in Flagstaff, Arizona (35.18418°N, 111.63294°W (DD)) and Oak Creek, AZ near the Cave Springs campground (34.9961°N, 111.7394°W (DD)) were collected for molecular analyses. These specimens were also fixed in 95% ethanol.

Morphological examination

We documented number of eyes and their placement, color pattern, presence of papillae, number of and structure of gastric caecae, body size, presence of nuchal scute, gonopore placement, egg size and number, and number of offspring using a Nikon binocular dissecting microscope. We then deposited the examined materials in the Invertebrate Zoology collection at the Smithsonian Institution, National Museum of Natural History (USNM).

Molecular analysis

Whole DNA was extracted from the caudal suckers of the individual leeches using a Qiagen DNeasy Blood & Tissue Kit (Cat. No. 69504), with each sample incubated

Table 1. *Helobdella* and outgroup taxa used for our molecular analysis.

Taxon	Locality	Reference
<i>Cystobranchus salmositicus</i>	Outgroup	Williams and Burreson 2006
<i>Ozobranchus margoi</i>	Outgroup	Siddall and Burreson 1998
<i>Gonimosobdella klemmi</i>	Outgroup	Williams and Burreson 2005
<i>Myzobdella lugubris</i>	Outgroup	Siddall and Burreson 1998
<i>Helobdella atli</i>	French Guiana	Oceguera-Figueroa et al. 2010
<i>Helobdella atli</i>	Uruguay	Oceguera-Figueroa et al. 2010
<i>Helobdella atli</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella blinni</i> sp. n.	Montezuma Well, AZ, USA	This study
<i>Helobdella blinni</i> sp. n.	Montezuma Well, AZ, USA	This study
<i>Helobdella blinni</i> sp. n.	Montezuma Well, AZ, USA	This study
<i>Helobdella bolivianita</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella bowermani</i>	Oregon, USA	Moser et al. 2013
<i>Helobdella bowermani</i>	Oregon, USA	Moser et al. 2013
<i>Helobdella bowermani</i>	Oregon, USA	Moser et al. 2013
<i>Helobdella californica</i>	California, USA	Kutschera 2011
<i>Helobdella "elongata"</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella europaea</i>	Taiwan	Lai et al. 2009
<i>Helobdella europaea</i>	Taiwan	Lai et al. 2009
<i>Helobdella europaea</i>	Taiwan	Lai et al. 2009
<i>Helobdella europaea</i>	Taiwan	Lai et al. 2009
<i>Helobdella europaea</i>	South Africa	Siddall and Budinoff 2005
<i>Helobdella lineata</i>	Michigan, USA	Siddall and Borda 2002
<i>Helobdella fusca</i>	Michigan, USA	Siddall and Borda 2002
<i>Helobdella melananus</i>	Taiwan	Lai et al. 2009
<i>Helobdella melananus</i>	Taiwan	Lai et al. 2009
<i>Helobdella melananus</i>	Taiwan	Lai et al. 2009
<i>Helobdella michaelsoni</i>	Chile	Siddall and Borda 2002
<i>Helobdella modesta</i>	Columbus, Ohio, USA	Siddall and Borda 2002
<i>Helobdella modesta</i>	Washington, USA	Oceguera-Figueroa et al. 2010
<i>Helobdella modesta</i>	Washington, USA	Oceguera-Figueroa et al. 2010
<i>Helobdella</i> c.f. <i>modesta</i>	Rio de Flag, Flagstaff, AZ, USA	This study
<i>Helobdella</i> c.f. <i>modesta</i>	Rio de Flag, Flagstaff, AZ, USA	This study
<i>Helobdella</i> c.f. <i>modesta</i>	Oak Creek, AZ, USA	This study
<i>Helobdella</i> c.f. <i>modesta</i>	Oak Creek, AZ, USA	This study
<i>Helobdella nunununuojensis</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella nunununuojensis</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	Taiwan	Lai et al. 2009
<i>Helobdella octatestisaca</i>	South Africa	Oceguera-Figueroa et al. 2010

Taxon	Locality	Reference
<i>Helobdella octatestisaca</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella octatestisaca</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella octatestisaca</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella octatestisaca</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella octatestisaca</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella papillata</i>	Michigan, USA	Siddall and Borda 2002
<i>Helobdella papillata</i>	Virginia, USA	Siddall and Borda 2002
<i>Helobdella papillornata</i>	Australia	Siddall and Borda 2002
<i>Helobdella paranensis</i>	Uruguay	Siddall and Borda 2002
<i>Helobdella pichipanan</i>	Bolivia	Siddall et al. 2005
<i>Helobdella</i> “robusta”TXAU1	Texas, USA	Bely and Weisblat 2006
<i>Helobdella</i> “robusta”	California, USA	Bely and Weisblat 2006
<i>Helobdella</i> “robusta”CASA 1	California, USA	Bely and Weisblat 2006
<i>Helobdella</i> “robusta”NYTA	New York, USA	Bely and Weisblat 2006
<i>Helobdella simplex</i>	Argentina	Moser et al. 2006
<i>Helobdella simplex</i>	Argentina	Moser et al. 2006
<i>Helobdella simplex</i>	Argentina	Moser et al. 2006
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella socimulcensis</i>	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella</i> sp. Xochimilco	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella sorojchi</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella sorojchi</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella stagnalis</i>	United Kingdom	Siddall and Borda 2002
<i>Helobdella</i> “stagnalis”	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella</i> “stagnalis”	Mexico	Oceguera-Figueroa et al. 2010
<i>Helobdella transversa</i>	Michigan, USA	Siddall and Borda 2002
<i>Helobdella triserialis</i>	Bolivia	Siddall and Borda 2002
<i>Helobdella triserialis</i>	California, USA	Bely and Weisblat 2006
<i>Helobdella virginiae</i>	Mexico	Oceguera-Figueroa et al. 2010

overnight in a water bath set at 54°C. Using Siddall and Borda’s (2002) PCR method, the mitochondrial gene region, cytochrome *c* oxidase subunit I (COI) was amplified. The primers were LCO1490 5’-GGTCAACAAATCATAAAGATATTGG-3’ and HCO2198 5’-TAAACTTCAGGGTGACCAAAAAATCA-3’ (Folmer et al. 1994). The PCR product was purified through the use of the QIAquick PCR Purification Protocol (Cat. No. 28104), checked for PCR product using gel electrophoresis, and sequenced with an ABI Prism 3730 sequencer (Applied Biosystems). We imported the seven “cleanest” sequences and 71 comparative sequences (Table 1) from previous studies (Siddall and Burreson 1998, Siddall and Borda 2002, Siddall and Budinoff

2005, Siddall et al. 2005, Williams and Bureson 2005, Bely and Weisblat 2006, Williams and Bureson 2006, Lai et al. 2009, Oceguera-Figueroa et al. 2010, Kutschera 2011, Moser et al. 2013) from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) into MEGA7.0.18 (Kumar et al. 2016). We aligned the sequences automatically using MUSCLE (Edgar 2004) and then corrected the alignments by hand. We partitioned the data and performed the substitution model test by codon in Partitionfinder (Lanfear et al. 2012). The best substitution model test was General Time Reversal (GTR) +gamma which we used in our maximum-likelihood (ML) analysis (Lanave et al. 1984, Tavare 1986, Rodriguez et al. 1990). For ML analysis, we used RAxML v. 8 (Stamatakis 2014) and included 1,000 nonparametric bootstrap replicates. We used MrBayes for Bayesian inference analysis with ten million generations with a 25% burn-in and our support was assessed based on clade posterior probabilities (Ronquist and Huelsenbeck 2003). These analyses were conducted through CIPRES (Miller et al. 2010). We used PAUP* 4.0 (Swofford 2003) to construct parsimony phylogenies with 100 random additions. We performed the parsimony analysis twice, treating the deletions in the sequences as a 5th state and then as missing data. We performed an uncorrected p-distance analysis to examine nucleotide differences between sequences with 1,000 replicates in MEGA7.0.18 (Kumar et al. 2016).

Results

Family Glossiphoniidae Vaillant, 1890

Genus *Helobdella* Blanchard, 1896

Helobdella blinni sp. n.

<http://zoobank.org/B1B3D234-BC3F-4126-BF25-52DA00BA7EB9>

Figs 2, 3, 4

Type materials. Holotype. USNM 1186106 (Table 2).

Additional materials. Paratypes. (14 specimens) (USNM 1186107, 1186108, 1186109, 1186110, 1186111, 1186112, 1186113, 1186114, 1186115, 1186116, 1186117, 1186118, 1186119, 1186120) (Table 2)

Type locality. USA, Arizona: Yavapai County, Montezuma Well (34.6491°N, 111.7522°W (DD)), aquatic system, under rocks, 10 June 2012, R.K. Beresic-Perrins.

Etymology. We have named this new species, *Helobdella blinni* in honor of Dr. Dean W. Blinn for his dedication to natural history research at Montezuma Well. For over 20 years at Northern Arizona University, Dr. Blinn studied a wide range of organisms and their interactions at Montezuma Well including predator-prey interactions between *Motobdella montezuma* and the endemic amphipod, *Hyaella montezuma* Cole & Watkins, 1977.

Description. External morphology. Length of specimens 11 to 22 mm (mean + SE 16.6 + 3.2 N=24) and width 3 to 8 mm (5.7 + 1.1 N=28) (Table 3, Figs 2, 3).

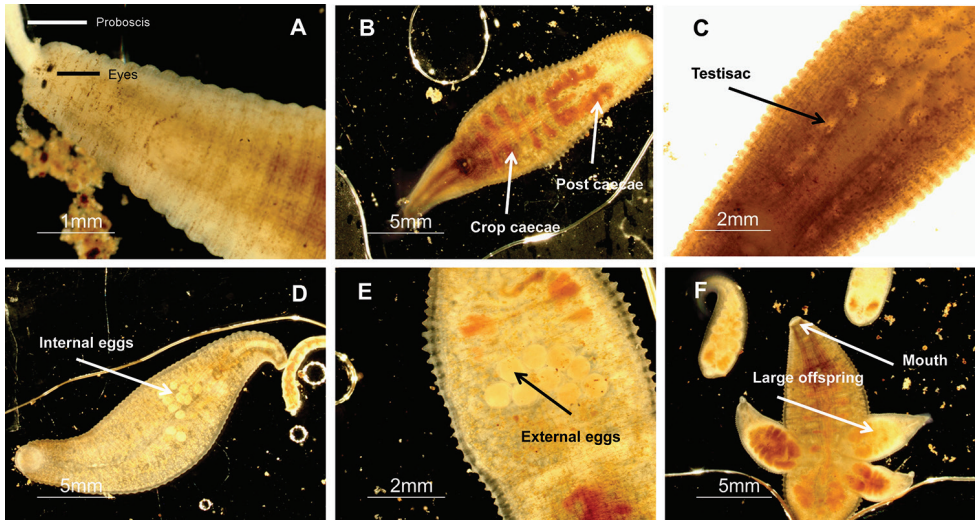


Figure 2. Internal and external morphology of *Helobdella blinni* sp. n. **A** dorsal view of the eyes and extended proboscis **B** crop and post caecae **C** testisacs **D** ventral view of internal eggs which have not been oviposited yet **E** ventral view of white eggs that have been oviposited **F** ventral view of attached and detached offspring.

Table 2. Holotype and paratype collection data and voucher numbers.

Family	Species	Collection data	Voucher #
Glossiphoniidae	<i>Helobdella blinni</i> sp. n.	USA: AZ: Yavapai Co., Montezuma Well 34.6491°N, 111.7522°W (DD), 10.VI.2010, aquatic system, under rocks, RK Beresic-Perrins, Holotype (USNM)	1186106
Glossiphoniidae	<i>Helobdella blinni</i> sp. n.	(14 specimens) USA: AZ Yavapai Co., Montezuma Well 34.6491°N, 111.7522°W (DD), 10.VI.2010, aquatic system, under rocks, RK Beresic-Perrins, Paratypes (USNM)	1186107
			1186108
			1186109
			1186110
			1186111
			1186112
			1186113
			1186114
			1186115
			1186116
			1186117
1186118			
1186119			
1186120			

Individual color ranges from translucent with brown spots to dark brown (Fig. 4). No dorsal papillae; one pair of eyes located at somite II (0.07 + 0.02 mm diameter, N = 11), distance between eyes 0.1 to 0.03 mm apart (N = 13). A scallop-shaped

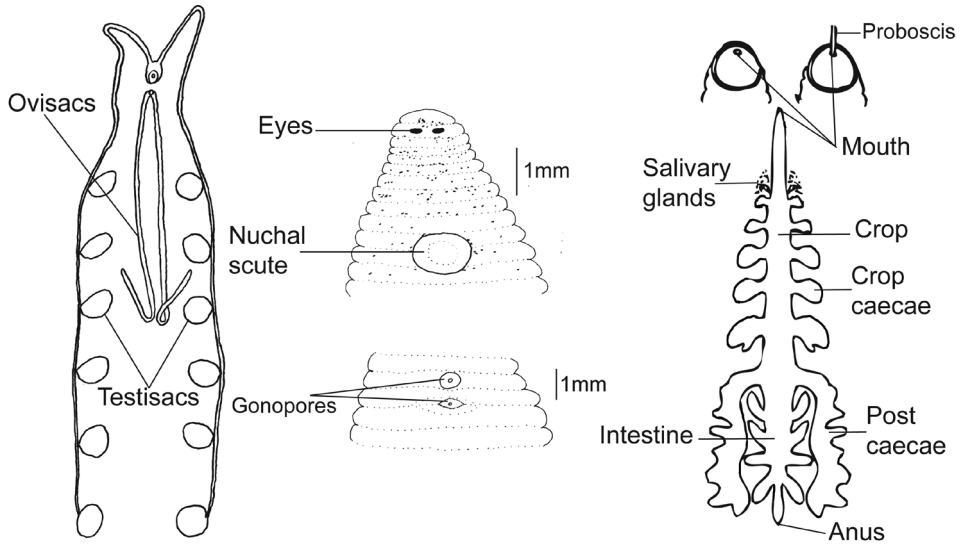


Figure 3. Diagram of the external and internal morphology of *Helobdella blinni* sp. n. (drawn by Rebecca Beresic-Perrins and Fredric Govedich).

Table 3. Morphological measurements of *Helobdella blinni* sp. n.

Trait	Ave	SE	Min	Max	N
body length relaxed (mm)	16.6	3.18	11.3	22.5	24
body width relaxed (mm)	5.7	1.15	3.1	8.0	28
caudal diameter (mm)	1.7	0.3	1.0	2.3	27
egg diameter (mm)	0.5	0.15	0.2	0.7	28
gonopore separation (mm)	0.1	0.08	0.1	0.3	13
nuchal scute length (mm)	0.335	0.05	0.284	0.432	9
nuchal scute width (mm)	0.32	0.04	0.27	0.386	9
proboscis length (mm)	3.5	1.10	2.0	6.2	17
oral sucker diameter (mm)	0.7	0.19	0.4	1.0	15
progeny length (mm)	3.6	1.68	1.6	6.6	18
progeny width (mm)	1.5	0.8	0.7	2.9	18
# eggs	10.0	2.73	7.0	16.0	7
# progeny	7.2	3.35	1.0	14.0	97
eye diameter (mm)	0.1	0.02	0.0	0.1	11
eye distance (mm)	0.1	0.04	0.0	0.2	13

nuchal scute is present on the dorsal side, length 0.293 to 0.432 mm ($0.335 + 0.05$ N=9) and width 0.27 to 0.386 mm ($0.32 + 0.04$ N=9). One annulus separates the female and male gonopores. The caudal sucker diameter averages $1.6 + 0.3$ mm (N = 27). The eggs (diameter $0.5 + 0.15$ mm, N = 28) are laid on the ventral side of the parent in soft-walled transparent cocoons (7–11 eggs per cocoon, N = 7). The mouth is located subterminally in the oral sucker (Figs 2, 3).



Figure 4. Typical pigmentation of *Helobdella blinni* sp. n.

Internal morphology. Average oral sucker diameter is $0.7 + 0.19$ mm ($N = 15$), proboscis length is $3.5 + 1.1$ mm ($N = 17$) (Table 3). Diffuse salivary glands are located near the anterior of the first pair of crop caecae. There are five pairs of smooth crop caecae and one lobed pair of posteriorly directed post caecae. Six pairs of compact testisacs are located in between each of the crop caecae. The intestine contains four pairs of caecae, with the first two pairs anteriorly directed and the other two pairs posteriorly directed. The intestine leads into an unraised anus located two annuli from the caudal sucker (Figure 3).

Development and growth. This species breeds year-round with peaks in spring and fall. Our specimens had an average of 7 to 11 white eggs (diameter $0.5 + 0.15$ mm, $N = 7$) fixed to their ventral surface. Laboratory collections (2007–10) of *H. blinni* documented the eggs hatching 1 to 2 weeks after ovipositing (Beresic-Perrins 2010). Once hatched, the young attach to the ventral surface of the parent, allowing the parent to hunt for food and feed the young, occasionally feeding along with them. Prey consists of oligochaetes and other invertebrates. The average number of young per

adult is $7 + 3.3$ ($N = 97$) ranging from 1 to 14 offspring. The young remain attached to the parent for an additional four to five weeks after hatching. Once the juveniles leave the parent, they tend to aggregate together on rocks (Beresic-Perrins 2010).

Molecular analysis

A Bayesian inference phylogenetic tree of the COI sequence data is presented in Figure 5. We include the posterior probabilities and maximum-likelihood branch supports >50 . The Arizona populations of *Helobdella* c.f. *modesta* formed a sister clade to *Helobdella blinni* sp. n., supported by both the Bayesian and parsimony analyses. The results of the uncorrected p-distance analysis revealed a difference of 13.3% (233 nucleotides included) between the two groups (Table 6). The two groups form a larger clade with *H. modesta* (Ohio), *H. stagnalis* (UK), and *H. modesta* (Washington) which is supported by both Bayesian inference and maximum-likelihood. *H. blinni* differed from *H. modesta* (Ohio) by 13.7%, *H. stagnalis* (UK) by 16.3%, and *H. modesta* (Washington) by 16.3% (Table 6).

When we aligned all 78 sequences, there were four, ten-codon deletions within all of the Arizona sequences and *H. atli* (Oceguera-Figueroa and León-Regagnon 2005, Oceguera-Figueroa et al. 2010). When we performed the parsimony analysis, we included deletions as a 5th state in our first analysis and in our second, we treated the deletions as missing data. In the resulting 5th state tree, the two Arizona species remained sister taxa (100% support), but included in the clade was *H. atli* (100% and 58% support). The missing data tree placed *H. blinni* ancestral to *H. modesta* (Washington), *H. modesta* (Ohio), *H. stagnalis* (UK), and *H. c.f. modesta* with 100% branch support (Fig. 5).

Discussion

Helobdella blinni sp. n. has morphological and life-history traits similar to other *Helobdella* species, including possession of a nuchal scute, diffuse salivary glands, six pairs of testisacs, and extended parental care for the young (6–7 weeks; Tables 4, 5). *Helobdella blinni*, *H. bowermani* (Moser et al. 2013), *H. octatestisaca* (Lai et al. 2009), and *H. c.f. modesta* each have five pairs of smooth crop caecae as opposed to six pairs of lobed crop caecae in *H. californica* (Kutschera 2011) and *H. papillornata* (Govedich and Davies 1998). *Helobdella blinni* and *H. temiscoensis* (Salas-Montiel et al. 2014) share pigmentation characteristics, but they differ internally. *Helobdella temiscoensis* has only four pairs of crop caecae and one descending post caecae as opposed to five pairs and one descending post caecae in *H. blinni*. *Helobdella modesta*, *H. californica*, *H. atli*, *H. bowermani*, and *H. octatestisaca* do not resemble the pigmentation of *H. blinni*, running the spectrum from grey to pink. Additionally, they have a descending pair of post caecae, whereas *H. atli*, *H. californica*, and *H. papillornata* do not. *Helobdella blinni*,

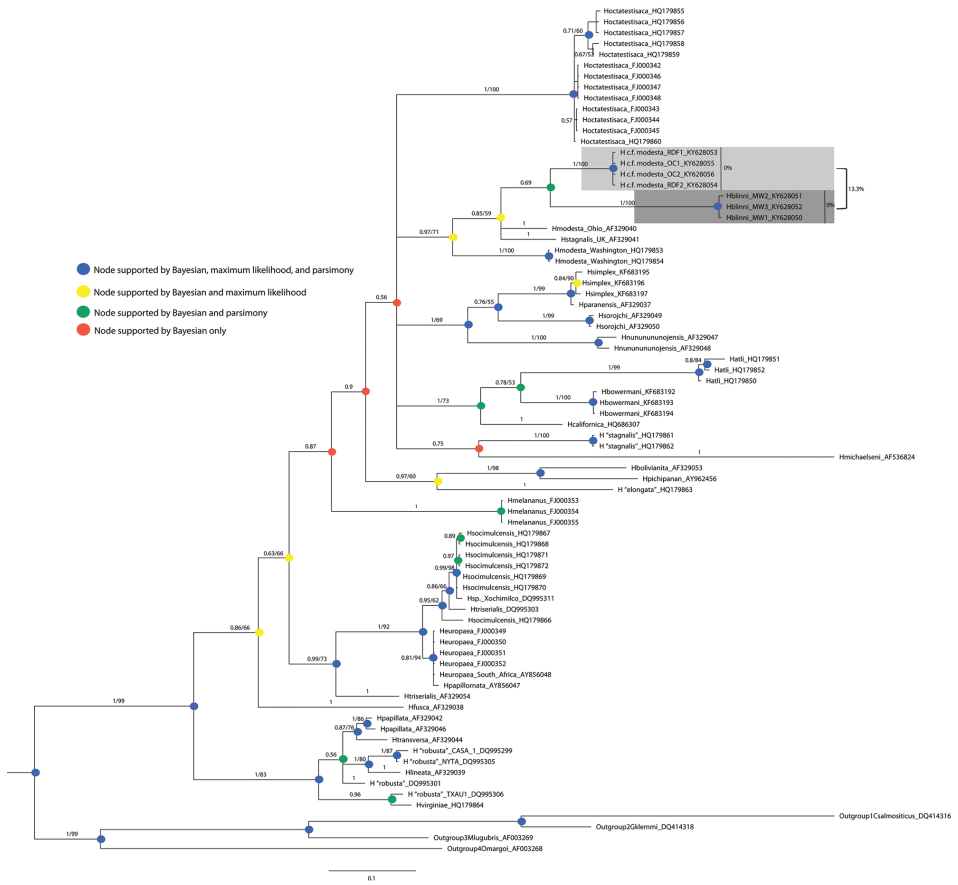


Figure 5. Bayesian Inference phylogenetic tree with 25% burn-in and support was assessed based on clade posterior probabilities tree. We included COI sequences from 31 species of *Helobdella* (family Glossiphoniidae). The Arizona populations are from Oak Creek (OC), Rio de Flag (RDF), and Montezuma Well (MW). Our outgroup included *Cystobranchnus salmositicus* (Meyer, 1946), *Gonimosobdella klemmi* (Williams & Burreson, 2005), *Myzobdella lugubris* (Leidy, 1851), and *Ozobranchnus margo* (Davies, 1978). The shaded branches are the Arizona sample sequences. Branch labels include the Bayesian / ML probability. The blue nodes are supported by Bayesian Inference, Maximum-Likelihood, and parsimony analyses. The yellow nodes are supported by Bayesian Inference and Maximum-Likelihood analyses. The green nodes are supported by Bayesian Inference and parsimony analyses. The red nodes are supported by Bayesian Inference analysis only.

H. c.f. modesta, *H. californica*, *H. temiscoensis*, *H. atli*, and *H. bowermani* possess six pairs of testisacs, whereas *H. papillornata* has five pairs and *H. octatetisaca* has four pairs. *Helobdella blinni* also has a larger proboscis than the other *Helobdella* species (mean + SE, *H. blinni* 3.5 mm + 1.1, N=17, *H. californica* mean = 0.7 mm, *H. papillornata* mean= 2 mm). Furthermore, breeding periods also differ between *H. blinni* and the other *Helobdella* species (Tables 4, 5).

Table 4. Morphological comparison of *Helobdella* species

Traits	<i>H. blinni</i> sp. n. (current paper)	<i>H. modesta</i> (Kuschera 1988; Sawyer 1986)	<i>H. californica</i> (Kuschera 1988; 2011)	<i>H. papillornata</i> (Govedich and Davies 1998)	<i>H. temiscoensis</i> (Salas-Montiel et al. 2014)	<i>H. atli</i> (Ocegüera-Figueroa and León-Regagnon 2005)	<i>H. bowermani</i> (Moser et al. 2013)	<i>H. octatesisaca</i> (Lai et al. 2009)
crop caecae	5 pairs, smooth	5 pairs, smooth	6 pairs, lobed	5–6 pairs, lobed	4 pairs	6 pairs	5 pairs, smooth	5 pairs
post caecae	1 pair	1 pair	none	none	1 pair	none	1 pair	1 pair
eyes	1 pair	1 pair	1 pair	1 pair)	1 pair	1 pair	1 pair	1 pair
distance between eyes	0.1 mm	?	?	0.06 mm	?	?	?	?
nuchal scute	yes	yes	yes	no	yes	yes	yes	yes
pairs of testisacs	6 pairs	6 pairs	6 pairs	5 pairs	6 pairs	6 pairs	6 pairs	4 pair
salivary glands	diffuse	diffuse	?	diffuse	diffuse	?	diffuse	diffuse
proboscis length	3.5mm	?	0.7mm	2mm	?	?	?	?
color	transparent with spots to dark brown	transparent to light grey	dark grey	transparent with stripes and papillae	pale brown, blackish - on posterior and mid-body	white-yellowish	pale yellow/ buff; papillae present	brown, pale, gray, and pink
body length	11–22 mm	8–12 mm	10–18 mm	15–40 mm	7.9–13.6 mm	7.5 mm	5.2–9.7 mm	9–14 mm
feeding	small invertebrates	small invertebrates	small invertebrates	small invertebrates	?	?	?	small invertebrates
brooding period	6–7 weeks	6–7 weeks	3–4 weeks	4–6 weeks	?	?	?	?
egg color	white	pink	pink	pink	?	?	?	?
egg diameter	0.5 mm	?	0.5 mm	0.2 mm	?	?	?	?
# eggs	7–14	12–35	8–56	20–50	?	?	?	?

Table 5. Differences in brooding season and size between *H. blinni* sp. n., *H. stagnalis*, and *H. c.f. modesta*.

Location	Brooding Season	Average # of offspring	Author
<i>H. blinni</i> sp. n. Montezuma Well, AZ	Year-round	1–14	Beresic-Perrins (2010)
<i>H. modesta</i> Utah Lake, UT	Late spring through summer	12.6–17.4	Tillman and Barnes (1973)
<i>H. modesta</i> Lake Washington, WA	Spring and Summer	14.5	Thut (1969)
<i>H. modesta</i> Marion Lake, BC, CA	Spring and Summer	17.2–19.7	Davies and Reynoldson (1976)
<i>H. modesta</i> Newsome Pond, AB, CA	Late spring through summer	21.3	
<i>H. modesta</i> Cambridge, MA	Spring	31	Castle (1900)
<i>H. modesta</i> Michigan	Late spring through summer	35.3	Sawyer (1972)
<i>H. stagnalis</i> Iceland	Late spring through summer	No data	Bruun (1938)
<i>H. stagnalis</i> River Ely, South Wales	Late spring through summer	No data	Murphy and Learner (1982)
<i>H. stagnalis</i> Whitknights Lake, UK	Late spring through summer	13–17	Mann (1957)
<i>H. stagnalis</i> Eglwys Nunydd, UK	Late spring through summer	14	Learner and Potter (1974)
<i>H. stagnalis</i> Denmark	Late spring through summer	20	Bennike (1943)

Helobdella blinni, unlike the other *Helobdella* species discussed here, breeds year-round, living in the thermally stable environment of Montezuma Well, with constant (19–24°C) year-round temperatures (Table 5). Monthly samples have individuals carrying cocoons every month of the year, with peak seasons in the spring and fall, a situation quite different than that for other *Helobdella* species, which have seasonally-constrained reproductive cycles, with egg-laying and brooding beginning in the spring and ending in the fall every year (Table 5). In addition to breeding year-round, *H. blinni* produces smaller broods (7–14 young) when compared to *H. modesta* and *H. stagnalis* (12–35 young) (Tables 4, 5) and has white eggs, unlike the characteristically pink eggs of *H. modesta*, *H. californica*, and *H. papillornata* (Table 4). The external pigmentation of *H. blinni* also tends to be dark brown, whereas most other *Helobdella* species are grey/brown in color (Fig. 4). *Helobdella blinni* are slightly longer (body length 11–22 mm, 16.6 + 3.2, N=24) than *H. c.f. modesta* (8–12 mm) and *H. californica* (10–18 mm), but slightly shorter in length than *H. papillornata* (15–40 mm) (Table 4).

The results from our molecular analysis show *H. blinni* to be genetically distinct from other *Helobdella* species, both from the same region (Rio de Flag and Oak Creek,

Table 6. Uncorrected p-distance pairwise analysis.

Species	Distance - <i>H. blinni</i>	Distance - <i>H. c.f. modesta</i>
<i>H. atli</i>	14.1–15%	16.7%
<i>H. bolivianita</i>	18.5%	19.3%
<i>H. bowermani</i>	15.9%	15.5%
<i>H. blinni</i>	0.0%	13.3%
<i>H. californica</i>	16.7%	17.6%
<i>H. c.f. modesta</i>	13.3%	0.0%
<i>H. elongata</i>	18.0%	19.3%
<i>H. europaea</i>	15.5%	16.3%
<i>H. fusca</i>	19.3%	20.6%
<i>H. lineata</i>	16.3%	14.6%
<i>H. melananus</i>	16.3%	17.2%
<i>H. michaelsoni</i>	23.2%	20.6%
<i>H. modesta</i> OH	13.7%	8.6%
<i>H. modesta</i> WA	16.3%	14.6%
<i>H. nunununuojensis</i>	17.5–19%	17.5–19.7%
<i>H. octatestisaca</i>	19.7%	16.3%
<i>H. papillata</i>	17.6%	15.8–16.3%
<i>H. papillornata</i>	15.9%	16.7%
<i>H. paranensis</i>	16.3%	13.7%
<i>H. pichipanan</i>	17.2%	19.3%
<i>H. robusta</i>	17.6%	14.6%
<i>H. aff robusta</i> CASA	18.9%	17.2%
<i>H. aff robusta</i> NYTA	17.6%	15.9%
<i>H. aff robusta</i> TXAU1	17.2%	18.0%
<i>H. simplex</i>	16.3%	13.7–14.2%
<i>H. socimulcensis</i>	15.9%	16.7–17.2%
<i>H. sorojchi</i>	18–18.5%	17.6%
<i>H. sp.</i> Xochimilco	15.5%	17.2%
<i>H. stagnalis</i>	20.6%	17.2%
<i>H. stagnalis</i> UK	16.3%	11.6%
<i>H. transversa</i>	16.3%	15.0%
<i>H. triserialis</i>	15.9%	16.7–18.5%
<i>H. virginiae</i>	16.3%	16.7%
Outgroup1 <i>C. salmositicus</i>	24.9%	23.2%
Outgroup2 <i>G. klemmi</i>	21.5%	21.5%
Outgroup3 <i>M. lugubris</i>	18.0%	19.3%
Outgroup4 <i>O. margo</i>	23.2%	20.2%

Arizona populations) and from Europe (UK sample). The sequences yielded a 13.3%–17.4% genetic difference between *H. blinni* and both the Arizona *H. c.f. modesta*, *H. modesta* (Ohio and Washington), and the United Kingdom *H. stagnalis* populations (Table 6). The three Arizona populations belong to their own separate clade, but are closely related to *H. stagnalis* (UK) and the *H. modesta*. *Helobdella atli*, *H. bowermani*,

and *H. californica* are located on separate branch tips, but they comprise what Ocegüera-Figueroa et al. (2010) designated as the “*stagnalis*” series (Fig. 5).

Based on morphological, life-history, and molecular differences, we propose the *Helobdella* sp. leeches found at Montezuma Well should be considered a new species, likely the result of allopatric isolation. This concept supports our hypothesis that the leech species inhabiting Montezuma Well may have become isolated from other populations as far back as 11,000 years ago (Wagner and Blinn 1987). *Helobdella blinni* sp. n. can be considered a distinct species found in Montezuma Well and may also turn out to be endemic to the area. Further sampling and analyses are needed in order to verify endemism.

Although currently classified as *Helobdella* c.f. *modesta*, the Arizona populations from the Rio de Flag and Oak Creek may be an additional undescribed species based on our molecular analysis. Our next step is to investigate these populations more closely, comparing them to other local populations, including White Horse Lake and J.D. Dam Lake, AZ which also contain *H. modesta*.

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