Origin of the hungry caterpillar: Evolution of fasting in slug moths (Insecta: Lepidoptera: Limacodidae)

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ABSTRACT

Studies of caterpillar defense strategy evolution typically focus on aposematic coloration, gregarious behavior, and/or chemical defense. In the slug moth family Limacodidae, the evolution of chemical defense is coupled to the life history trait of first instar feeding behaviors. In nettle caterpillars, the first instars fast and molt into a second instar that feeds. In contrast, gelatinous and monkey slug larval forms feed in the first instar. This study focused on whether the evolution of fasting associated with the nettle morphology was a derived trait of single or multiple origins. Twenty-nine species of Limacodidae (including one Chrysopolominae) representing 27 genera and four outgroup species with known first and final instar morphologies and behaviors were included. Four out-group species representing Megalopygidae (1 sp), Dalceridae (1 sp) and Aididae (2 sp) were included. These were sequenced for three molecular markers fragments for a total of 4073 bp, mitochondrial COI (~1500 bp), 18S (~1900 bp) and the D2 region of 28S (approximately 670 bp). Maximum likelihood and Bayesian analyses were conducted. The resulting phylogeny and

comparative analysis of feeding strategy revealed that the nettle caterpillar morphology and behavior of larval fasting may have a single origin.

Keywords (3) –Zygaenoidea, larval ontogeny, mtDNA

1. Introduction

The slug moth family Limacodidae (1,650 species) (Nieukerken et al., 2011) has intrigued researchers for decades. Primarily tropical, they have colorful and often poisonous caterpillars with a bizarre array of forms and markings. Their curious larval locomotion is something to behold: their highly elastic underbelly moves in wavelike pulses and aided by a semifluid silk to stick to preferred smooth-leaved food plants or to grasp stems (Epstein, 1995; Lill et al., 2006).

The common name “slug moth” derives from “gelatine” caterpillars that resemble gelatinous slugs (Fig. 1E, Murphy et al., 2011). However, the majority of species have “nettle” caterpillar adorned with poisonous spines, known to cause dermatitis in humans (Fig. 1C; Hossler, 2010). A third, less common larval type, with “hairy” caterpillars is the ‘monkey slug’, which have many fine setae borne on elongated tubercles (Fig. 1I).

Most limacodid larvae undergo significant ontogenetic changes in morphology rather than hetero- or hypomorphic changes because they don’t involve “major shifts in biology” (Epstein, 1996). The largest change often occurs when molting from 1st to 2nd instar (Fig. 1). First instars of most species have segments with at least two pairs of protuberances per segment, each with one to three spines or fleshy setae. The 2nd instar molt results in one of the three divergent morphologies. Upon molting, nettles have a dramatic increase in spines (Fig. 1B). “Gelatines”, conversely, lose the protuberances and have a smooth appearance (Fig. 1E). Finally, “hairys” retain the first instar tubercles, which continue to elongate with progressive molts (Figs. 1H-I)(Epstein, 1996).

The three larval types also exhibit distinct feeding behaviors in the first instar. First instars of “gelatine” and “hairy” species feed on leaves as is typical of lepidopteran larvae (Figs. 1D, 1G)(Dyar, 1895-1914). In contrast, “nettles” have the curious habit of “fasting” for the entire first larval instar after hatching (Fig. 1A). First reported by Dyar and Morton (1895) in their life

history of the saddleback caterpillar (*Acharia stimulea*), this fasting behavior likely occurs in nearly all limacodid species with nettle caterpillars based upon our current knowledge of genera with this larval form (Dyar, 1899; Nagamine and Epstein, 2007; Murphy et al., 2011; Epstein, pers observ.).

Some researchers suggest that the defensive strategy of “stinging” in nettle caterpillars confers a significant adaptive advantage in the species that have recruited this defensive strategy over the more cryptic “gelatine” defense (Murphy et al., 2010, 2011). Further, some have hypothesized that spines cannot be expressed in the neonate nettle larvae because the thin chorion of limacodid eggs would rupture (Nagamine and Epstein, 2007). Murphy et al. (2010, 2011) further suggested that other behaviors and morphologies observed by Dyar (1895-1914) such as laying eggs in large clusters, larval gregariousness and aposematic coloration co-evolved as a suite of traits that enhance the defensive strategy of spiny larvae.

To determine the evolutionary origins of nettle caterpillars and fasting, a generic-level phylogeny is needed. In contrast to its well-established relationships within Lepidoptera, Limacodidae lacks a well-corroborated phylogeny for its genera and generic groups. The first depiction of limacodid evolution was based on characteristics of the caterpillars, including the first instars (Dyar, 1899). More recent phylogenetic research on the moth family has been geographically restricted and focused on the genus *Parasa* and related genera (e.g., Epstein, 1988; Solovyev, 2014). Morphologically based phylogenetic research on the placement of Limacodidae supported the taxonomic concept of a “Limacodid group” consisting of the Limacodidae, Dalceridae, Aididae, and Megalopygidae within the superfamily Zygaenoidea (Epstein 1996, Epstein, et al. 1999). Molecular studies, including Regier et al. 2009, support an expanded Limacodid group, however, taxon sampling has not focused on many genera of the Limacodidae.

A well-supported phylogeny for Limacodidae is necessary to examine patterns of larval behavior and development. Here we reconstruct the evolutionary relationships among limacodid genera at a more global level to illuminate the evolution of this unique fasting behavior within the lineage.

2. Materials and Methods

2.1 Taxon sampling and DNA sequencing

The taxa included in this study were selected primarily by the availability of immature stages for parallel studies on morphology. Adult moths were sampled at lights and placed in at least 95% ethanol in the field; later specimens were frozen and maintained at -20°C. Data on the immatures came from eggs obtained from gravid females collected at lights (Table 1) and terminology used for larval morphology follows Epstein (1996). The ingroup was represented by 27 genera and 29 species of Limacodidae. Four outgroup species representing Megalopygidae, Dalceridae, and Aididae were also included in our analysis.

DNA extractions were performed on excised thoracic tissue or 1–2 legs from sample specimens. DNA extraction on all samples was performed using the DNEasy tissue extraction kit (QIAGEN) and following the manufacturer's protocol for animal tissues. The following three genetic markers were amplified based on previous studies demonstrating their usefulness in resolving evolutionary relationships among lepidopteran insects above and below the family level: (1) D2 region of the 28S ribosomal subunit sequence, (Lee and Brown 2008) (2) COI gene (Simon et al. 1994) and (3) 18S (Chalwatzis et al. 1995, Niehuis et al. 2006); a complete list of primer sequences is given in Supplemental Materials (Table 3). Polymerase chain reactions (PCR) for COI were performed in 50µL volumes using the following recipe: 0.3 µL TaKaRa Ex *Taq* polymerase, 5.0 µL Takara 10x buffer, 4.0 µL TaKaRa dNTPs (2.5mM of each nucleotide), 2.0 µL

of each primer (10 μ M), 2.0 μ L template DNA (3.0 μ L for difficult samples), and ddH₂O to total 50.0 μ L. For 18S and 28S, reactions were prepared in 25 μ L volumes as follows: 12.5 μ L ACCUZYME Mix (Bioline), 0.5 μ L *Taq* polymerase (Bioline, USA), 1.0 μ L of each primer (10 μ M), 1.0 μ L DNA template, and 9.0 μ L ddH₂O. Successfully amplified PCR products were purified using a QIAquick PCR Purification kit (QIAGEN, USA), following the manufacturer's protocol. The PCR cycling profile for 18S was 94°C for 180 s, followed by 15 cycles of (94°C for 35 s, 55-40°C for 30 s, and 72°C for 90 s). For the first 15 cycles, the annealing temperature was reduced by 1°C each cycle, starting at 55°C and ending at 40°C. Cycling was continued for an additional 25 cycles at an annealing temperature of 50°C, followed by a final extension at 72°C for 600 s. The PCR cycling profile for 28S was 94°C for 150 s, 40 cycles of (94°C for 30 s, 65°C for 30 s, and 72°C for 30 s), and a final extension at 72°C for 120 s. The PCR cycling profile for COI was 95°C for 540 s, 34 cycles of (94°C for 60 s, 49°C for 60 s, and 72°C for 60 s), and a final extension at 72°C for 180 s. Successful PCR amplification was verified by submitting products to agarose gel electrophoresis, SYBR Safe DNA Gel Stain (Life Technologies, USA) to visualize DNA. Purified PCR products were sequenced using an ABI 3730xl (Applied Biosystems, USA) using ABI BigDye v. 3.1 Terminator chemistry (PE Applied Biosystems, USA) at the BioMedical Genomics Center (BMGC) at the University of Minnesota, St. Paul campus.

2.2. Sequence alignment and phylogenetic analyses

Verification of base calls, comparison of complimentary chromatograms, and trimming of primers were performed manually using Geneious R6 v.6.1.6 (<http://www.geneious.com>, Kearse et al., 2012). DNA sequence alignments were generated using MAFFT v.7.017 implemented in Geneious R6 v.6.1.6. Neighbor-joining and Bayesian analyses of single-gene alignments were

performed to test for rogue sequences. In some instances, the sequence amplified but sequenced poorly. If attempts to procure better sequence were unsuccessful, questionable data were omitted. Maximum likelihood (ML) and Bayesian Inference (BI) analyses were carried out on the combined data set. The Bayesian Information Criterion (BIC) using Partition Finder v. 1.1.1 (Lanfear, 2012) was applied to determine the best partitioning scheme for codon positions in protein-coding sequences and corresponding best evolutionary models for the dataset (Supplemental Materials Table 2). A maximum likelihood (ML) analysis was performed using RAxML-HPC BlackBox v. 7.4.4 on the CIPRES Web Portal at [http:// www.phylo.org/portal2/](http://www.phylo.org/portal2/) (Miller et al., 2010). Bootstrapping runs (1,000 replicates) to calculate ML nodal support were also performed on the CIPRES site using the RAxML-HPC BlackBox utility. A partitioned Bayesian analysis of 2,000,000 generations, with 25% of trees discarded as burn-in, was also carried out using the MrBayes v.3.2.2 on XSEDE utility on the CIPRES Web Portal at <http://www.phylo.org/portal2/> (Miller et al., 2010).

2.3 Ancestral state reconstruction

First instar-feeding behaviors differ in whether emerging larvae feed immediately or whether they “fast” and only commence feeding once they have molted to second instar (Dyar and Morton, 1895; Nagamine and Epstein, 2007). Corresponding larval protective ornamentation (spiny, smooth, tubercles, dehiscent tubercles, droplets) has been well documented in the Limacodidae and related Limacodid-group families (e.g., Epstein, 1996; Murphy et al., 2011). These traits were divided into the following categories and coded for terminal taxa included in the ML analysis. The ingroup traits are A-F (all 1st instars feed upon hatching except E) and outgroup traits are G,H and I (all feed): (A) “hairy” (tubercles remain in all instars, but dehisce after first instar when pulled and

when building cocoon); (B) “gelatine” (tubercles not formed after 1st or 2nd instar); (C) “gelatine” (tubercles not formed after 1st or 2nd instar, secrete droplets on dorsum in late instars); (D) “gelatine” (wartlike tubercles in first instar do not form in 2nd or following instars); (E) fasting “nettle” (many spines borne on scoli after first instar molts to 2nd instar); (F) “gelatine” (no tubercles in 1st instar and do not change form in remaining molts); (G) “gelatine” (tubercles remain after first instar, but secrete slime beneath cuticle and dehisce after 1st instar); (H) “nettle” (a single spine on dorsal tubercles, spines increase after first instar); (I) “nettle” (many spines borne on verrucae from first instar on). A matrix with character codings for these traits is provided in Supplemental Materials Table 1. Probabilities of each category were calculated using the Bayesian Binary MCMC (BBM) method implemented in RASP (Yu et al., 2015).

3. Results and Discussion

Our phylogenetic analyses were based on sequence data from three molecular markers fragments for a total of 4073 bp: mitochondrial COI (~1500 bp), 18S (~1900 bp) and the D2 region of 28S (approximately 670 bp) for 33 terminal taxa. A complete list of amplified markers and voucher information for each taxon is provided in Table 1. Optimal topologies found by BI and ML for combined datasets were relatively congruent, and all strongly supported clades in the ML analysis also recovered in the BI tree (compare Fig. 2 and Supplemental Fig. 1).

Phylogenetic results from the ML analysis and the likely results from the RASP analysis are shown in Fig. 2. The recovered evolutionary pattern of larval morphology and first instar behavior suggests that the “fasting” behavior is derived (Node 4: BS=77). Researchers (Nagamine and Epstein 2007, Murphy et al. 2011) suggest that fasting and the quick molt to become spinier was a derived condition that evolved in response to the limacodid egg-type. Ancestral groups

Megalopygidae and Aididae have thick chorions, raised eggs; hatching larvae are spiny and feed immediately. In addition to fasting, “nettles” tend to lay eggs in clusters and larvae are gregarious whereas “gelatines” lay eggs singly and are solitary.

The monophyly of Limacodidae, minus *Pantoctenia*, was recovered in both maximum likelihood and Bayesian analyses although support was not significant. However, with the exclusion of *Pantoctenia prasina*, monophyly of Limacodidae was well supported in the Bayesian analysis (PP = .97). Epstein (1996) suggested *Pantoctenia* was sister to either Limacodidae or Dalceridae; our results provide additional support as sister to Dalceridae (Fig. 2). Within Limacodidae (Node 2: BS=75), all seven included generic complexes, considered to be monophyletic by Epstein (1996), were recovered, albeit without significant support in some cases, from the molecular data: *Miresa* (Node 7: BS=88), *Parasa* (Node 6: BS=58), *Natada* (Node 5: BS=99), *Phobetron* (Node 10: BS=11), *Prolimacodes* (Node 9: BS=10), *Apoda* (Node 11: BS=100), and *Perola* (both same genus) (Node 1: BS=100).

Another result was that the *Phobetron* complex (“hairy”, monkey slug caterpillars) was found to be sister to the *Prolimacodes* complex (“gelatine”). Although weaker in molecular support for this clade (Node 8: BS=59) than the “nettle” clade (Node 4: BS=77), morphological support exists for this association (Epstein, pers observ.). Contrarily, Dyar (1899) placed *Phobetron* at the base of “nettle” branch with *Parasa* and *Natada* complexes. Limacodids with “gelatine” morphology were placed in several clades, including *Perola* (Node 1: BS=100), which was found to be sister to all the remaining Limacodidae (Node 3: BS=78).

Placement of *Cryptophobetron oropeso* within the *Prolimacodes*+*Phobetron* clade was unexpected. Its first instar differs from other “gelatines” in this study by having few tubercles in first instars and late instars make no droplets like others in the *Prolimacodes* complex (Epstein,

pers observ.). The adults differ from other New World species as well, but do share similarly shaped adult female pretarsal pads with the *Apoda* complex (Epstein, 1996).

The only Chrysopolominae herein, *Strigivenifera venata* (a “gelatine”), and the Asian species *Darna pallivitta* (a “nettle”) each formed weakly supported clades with genera of similar larval types. Greater taxon sampling from Africa and Asia will likely group these taxa with others from their respective regions, while providing additional structure to the tree.

The hungry caterpillar strategy appears more prevalent in the New World tropics than temperate areas. It has been estimated that 62% of the 120 limacodid species in Costa Rica fast as 1st instars and the remainder feed, whereas only 46% of the 46 resident North American species are fasting “nettles” (Epstein, pers observ.). However, the members of the North American fauna from Neotropical lineages (30 species) have a similar percent of fasting to those in Costa Rica at 70%. The difference in feeding versus fasting in the two locations is explained by 16 species of feeding “gelatines” that share ancestry with Asian and Palearctic species (Dyar, 1899).

As has been noted in other lepidopteran molecular studies, multiple molecular markers will be needed to resolve limacodid relationships with statistical confidence below the subfamily level (Zaspel et al., 2014; Regier et al., 2013). Because branch support was generally low in our analyses, ancestral reconstructions should be interpreted with caution. However, we are confident that the results herein provide a solid basis for elucidating general trends within the Limacodidae and set the stage for future work on the evolution of fasting first instars: the *hungry caterpillar* strategy.

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Figure 1. Fasting or feeding first instar caterpillars of Limacodidae (Insecta, Lepidoptera) and change in spines in subsequent instars (all photographs by Kenji Nishida). **A)** *Acharia horrida* fasting “nettle” 1st instar on top of egg shells; **B)** *A. horrida* 2nd instar “nettle” feeding (note: increase in spines); **C)** *A. horrida* late instar “nettle” feeding on edge of leaf, left (note: concentration of spines on sides and ends characteristic of saddleback caterpillars); **D)**

Venadicodia caneti feeding 1st instar (note: tubercles on back and frass to left of caterpillar as evidence of feeding); **E**) *V. caneti* “gelatine” 2nd or 3rd instar (note: absence of protuberances); **F**) *V. caneti* “gelatine” with defensive droplets on dorsal ridge of abdomen; **G**) *Phobetron hipparchia* feeding 1st instar (note: feeding damage); **H**) *P. hipparchia* “hairy” 2nd instar (note: additional hairs and that the hairy tubercles can be removed); **I**) *P. hipparchia* late instar “hairy” (note: the “monkey slug” tubercles will be incorporated into the outer cocoon, so it looks much like the caterpillar).

Figure 2. Phylogenetic hypothesis for Limacodidae (Insecta, Lepidoptera) based on maximum likelihood (ML) analysis, along with outgroups. Bootstrap values are shown next to the branches. Bayesian ancestral state reconstruction analysis for Limacodidae optimized on the ML topology, implemented in the program RASP (Yan et al., 2015). Larval defensive strategy reconstructions with highest marginal probabilities are indicated at each node. **A** = “hairy”, tubercles elongate and single in 1st instar, remaining all instars and become hairy, but can be pulled off; **B** = “gelatine”, tubercles not formed after 1st or 2nd instar; **C** = “gelatine”, tubercles not formed after 1st or 2nd instar, secrete droplets on dorsum in late instar; **D** = “gelatine”, wartlike tubercles don’t form after 1st instar (no reconstruction); **E** = fasting, “nettle”; **F** = “gelatine”, no tubercles in first instar and do not change form in remaining molts; **G** = “gelatine”, tubercles remain after first instar, but secrete slime beneath cuticle and dehisce after 1st instar; **H** = “nettle”, a single spine on dorsal tubercles in 1st instar, spines increase in subsequent molts; **I** = “nettle”, many spines borne on verrucae from 1st instar onward.

Table 1. Complete list of specimens used in the phylogenetic analysis including the collection locality, sample IDs, and the gene fragments that were successfully Genbank accession numbers are listed by gene region.

Figure 1A-I
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Figure 2
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Table 1

Table 1. Complete list of specimens used in the phylogenetic analysis including the collection locality, sample IDs, and the gene fragments that were successfully amplified. Genbank accession numbers are listed in the last 3 columns by gene region.

	FAMILY	Subfamily	Genus species	Locality	Voucher	Gene regions		
						18S	28S	COI
INGROUP	Lima-	Chrysopolomina	<i>Strigivenifera venata</i>	Nigeria	SEM-06-5643	KR068957	KR068990	KR260966
		Limacodinae	<i>Acharia stimulea</i>	USA	MEE-98-0015	KR068930	KR068963	KR260939
			<i>Alarodia slossoniae</i>	USA	MEE-05-0006	KR068933	KR068966	KR260941
			<i>Apoda biguttata</i>	USA	UNK-95-1002	KR068934	KR068967	KR260942
			<i>Cryptophobetron oropeso</i>	USA	MEE-05-0025	KR068935	KR068968	KR260944
			<i>Darna pallivitta</i>	Asia/Hawaiian invasive	MEE-01-002	KR068937	KR068970	KR260946
			<i>Dichromapteryx didyma</i>	Costa Rica	MEE-05-0028	KR068938	KR068971	KR260947
			<i>Euclea delphinii</i>	USA	AM-94-0274	KR068939	KR068972	KR260948
			<i>Euprosteria elaea</i>	Costa Rica	MEE-01-003	KR068940	KR068973	KR260949
			<i>Isa textula</i>	USA	MEE-98-0032	KR068941	KR068974	KR260950
			<i>Isochaetes beutenmuelleri</i>	USA	AM-94-0279	KR068942	KR068975	KR260951
			<i>Miresa clarissa</i>	Costa Rica	MEE-05-0040	KR068944	KR068977	KR260953
			<i>Monoleuca semifascia</i>	USA	MEE-98-0017	KR068945	KR068978	KR260954
			<i>Narosoideus flavidorsalis</i>	Japan	A-1073	KR068946	KR068979	KR260955
			<i>Narosopsis iangauldi</i>	Costa Rica	MEE-05-0034	KR068947	KR068980	KR260956
			<i>Natada nasoni</i>	USA	MEE-05-0011	KR068948	KR068981	KR260957
			<i>Pantoctenia prasina</i>	Tanzania	MF-05-0084	KR068949	KR068982	KR260958
			<i>Parasa chloris</i>	USA	MEE-05-0013	KR068950	KR068983	KR260959
			<i>Parasa lepida</i>	Japan	AYK-04-5396	KR068951	KR068984	KR260960
			<i>Perola cicur</i>	Brazil	MEE-98-0082	KR068952	KR068985	KR260961
			<i>Perola murina</i>	Brazil	MEE-98-0077	KR068953	KR068986	KR260962
			<i>Phobetron pithecium</i>	USA	MEE-05-0010	KR068954	KR068987	KR260963
			<i>Prolimacodes badia</i>	USA	CWM-94-032	KR068955	KR068988	KR260964
			<i>Semyra coarctata</i> complex	Venezuela	MEE-98-0018	KR068956	KR068989	KR260965
			<i>Talima aurora</i>	Costa Rica	MEE-05-0052	KR068958	KR068991	KR260967
			<i>Tanadema neutra</i>	Costa Rica	MEE-05-0032	KR068959	KR068992	KR260968
			<i>Tortricidia pallida</i>	USA	MEE-98-0021	KR068960	KR068993	KR260969
			<i>Venadicodia caneti</i>	Costa Rica	05-srnp-1926	KR068961	KR068994	KR260970