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THE PROSTERNAL GLAND IN PACIFIC NORTHWEST BUTTERFLY LARVAE WITH PRELIMINARY
CHEMICAL ANALYSES OF EMISSIONS

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ABSTRACT. During rearing studies, larvae of 41 Pacific Northwest butterfly species from three families (Nymphalidae, Hesperidae and Pieridae) were identified as possessing a prosternal gland. Observations on larvae of *Argynnis* spp. (Nymphalidae) showed the gland appeared in the second instar as a pale-colored ventral suture. Rough handling of final instars caused eversion of a single-lobed papilla and emission of a 'musky' odor. The prosternal glands of all nymphalid and hesperid larvae examined were similar morphologically but the pierids, *Neophasia menapia* and *Nathalis iole* possessed a pair of bi-lobed glands. Chemical analyses revealed that the glands of final instar *Argynnis* spp. contained hydrocarbons, fatty alcohols, carboxylic acids and acetate esters. Dodecene or dodecanol and/or associated alkanes and acetate esters occurred in nearly all *Argynnis* samples as well as in the glands of *N. menapia* (Pieridae) and *Polites sonora* (Hesperidae) larvae. These compounds have a dispersive function in other arthropods. Terpenoid compounds were found in most samples and likely have a defensive function. Glands contained other potentially defensive compounds including disulfides, squalene, acridine, diphenyl ether and diphenylamine. Based on these preliminary data, the prosternal gland appears to have at least two functions in butterfly larvae: defense and dispersion. The apparent widespread occurrence of prosternal glands in larvae of Nymphalidae and Hesperidae suggests that this gland is important in the ecology of many species although experimental evidence for function is needed.

Additional key words: defense, dispersion, chemistry, hydrocarbons, acetate esters, terpenoids

The existence of a prosternal gland (also known as ventral eversible gland or adenosma) in some lepidopteran larvae was first reported by De Geer in 1745 (Latter 1897). Prosternal glands occur in larvae of Noctuidae, Notodontidae, Nymphalidae, Hesperidae and Pieridae according to Peterson (1962). Detailed anatomical and chemical studies on prosternal glands in lepidopteran larvae have been conducted for moths of the families Notodontidae and Noctuidae (Geertsema et al. 1976; Marti & Rogers 1988), where emissions from the gland have been suggested to have defensive (Weatherston et al. 1979; Severson et al. 1991) or dispersive (Weatherston et al. 1986) properties. Formic acid is the major component of prosternal gland secretions in *Schizura concinna* (J.E. Smith) larvae (Weatherston et al. 1979), but in another notodontid species, *Datana ministra* (Drury), secretions are dominated by the alkanol acetates, dodecanol, dodecyl acetate, and dodecyl formate (Weatherston et al. 1986). Prosternal gland volatiles from larvae of the noctuid *Spodoptera frugiperda* (J.E. Smith) contain saturated

hydrocarbons, primarily *n*-pentadecane (Severson et al. 1991). In butterflies, Scott (1986) briefly mentioned the occurrence of prosternal glands in some nymphalids, pierids and hesperiids. Muyschondt and Muyschondt (1976) and McCorkle and Hammond (1988) reported prosternal glands in larvae of *Colobura dirce* L. and *Speyeria* (= *Argynnis*) *zerene hippolyta* (Boisduval) (Nymphalidae), respectively. James (2008) described the presence of a prosternal gland in second to sixth instars of 5 *Argynnis* spp. from Washington State. Images of the gland in a sixth instar *A. coronis simaetha* (Behr) were also presented. Morphological studies on prosternal glands in *Abanante hylonome* (Doubleday) and *Heliconius erato* (L.) (Nymphalidae) were reported by Osborn et al. (1999) and Borges et al. (2010), respectively. To date, the only chemical analysis of prosternal glands in butterfly larvae was reported by Osborn and Jaffe (1998), who showed carboxylic acids and terpenes were present in prosternal gland secretions in the nymphalids *Dione juno* (Cramer) and *A. hylonome*.

This paper presents information on the occurrence of prosternal glands in larvae from three butterfly families in the Pacific Northwest. We also provide the results of preliminary chemical analyses of prosternal gland emissions mainly from *Argynnis* spp.

MATERIALS AND METHODS

During butterfly rearing studies in the Pacific Northwest during 2002–2010, observations were made on the presence or absence of prosternal glands in late instars of selected species of the families Pieridae, Nymphalidae and Hesperidae. Observations on gland appearance during larval development, size, eversion and color differences between species were made for *Argynnis* spp. (Nymphalidae). Some larvae were mounted, ventral surface up, restrained by sticky tape and photographs of glands were taken using a Canon EOS 1DS Mark II, digital SLR camera mounted on a tripod. A Canon MP-E 65 mm 1 X – 5 X macro lens was used together with a macro Twin Lite MT – 24 EX flash lighting system.

During 2007–2009, seventy eight extracts obtained from the prosternal glands of late instars of *Argynnis* (*Speyeria*) spp. (Nymphalidae) (76)), *Polites sonora* (Scudder) (Hesperidae) (1)) and *Neophasia menapia* (C. & R. Felder) (Pieridae) (1)) were analyzed using gas chromatography/mass spectrometry (GC/MS). Seven species of *Argynnis* (*Speyeria*) were examined in 2007: *A. (S.) zerene* (Boisduval), *A. (S.) coronis* (Behr), *A. (S.) hydaspe* (Boisduval), *A. (S.) hesperis* (Edwards), *A. (S.) egleis* (Behr), *A. (S.) cybele* (F.) and *A. (S.) mormonia* (Boisduval). *A. coronis*, *A. zerene* and *A. mormonia* were reexamined in 2008 as were *A. zerene* and *A. hydaspe* in 2009. *Argynnis* spp. were reared in the laboratory on *Viola adunca* Sm. (Blue Violet) and *V. labradorica* Schrank (Labrador Violet), after first instars in diapause were held at 5 °C for 2–3 months (James 2008). Gland extracts were taken from sixth instars. Single extracts were taken from a fourth instar *P. sonora* and a fourth instar *N. menapia* reared in the laboratory on Yellow Foxtail Grass (*Setaria glauca* (L.) P. Beauv) and Douglas Fir (*Pseudotsuga menziesii* Mirb. Franco), respectively. Extracts were obtained by restraining larvae, ventral side up, under a stereomicroscope and squeezing the anterior part of the body until the prosternal gland was everted. A small piece (~ 5 × 5 mm) of filter paper was held to the gland and fluid drawn off. The filter paper was deposited into a clean glass vial containing 0.5–2.0 ml of dichloromethane. Vials were stored in a freezer (1–2 weeks) until analyses using gas chromatography-mass spectroscopy (GC/MS) were conducted over the course of several days. Extracts were analyzed using 2µl

samples in an Agilent 6890N Gas Chromatograph with 5973N Mass Selective Detector (MSD) and an Agilent 7683 auto sampler. The carrier gas was ultrapure helium and the oven was held at 50 °C for 1 minute, raised to 260 °C at 5 °C /minute and held for 30 minutes. Masses between m/z 50 and 500 were scanned. Mass spectra were identified by comparison of retention times and mass spectra in the NIST library.

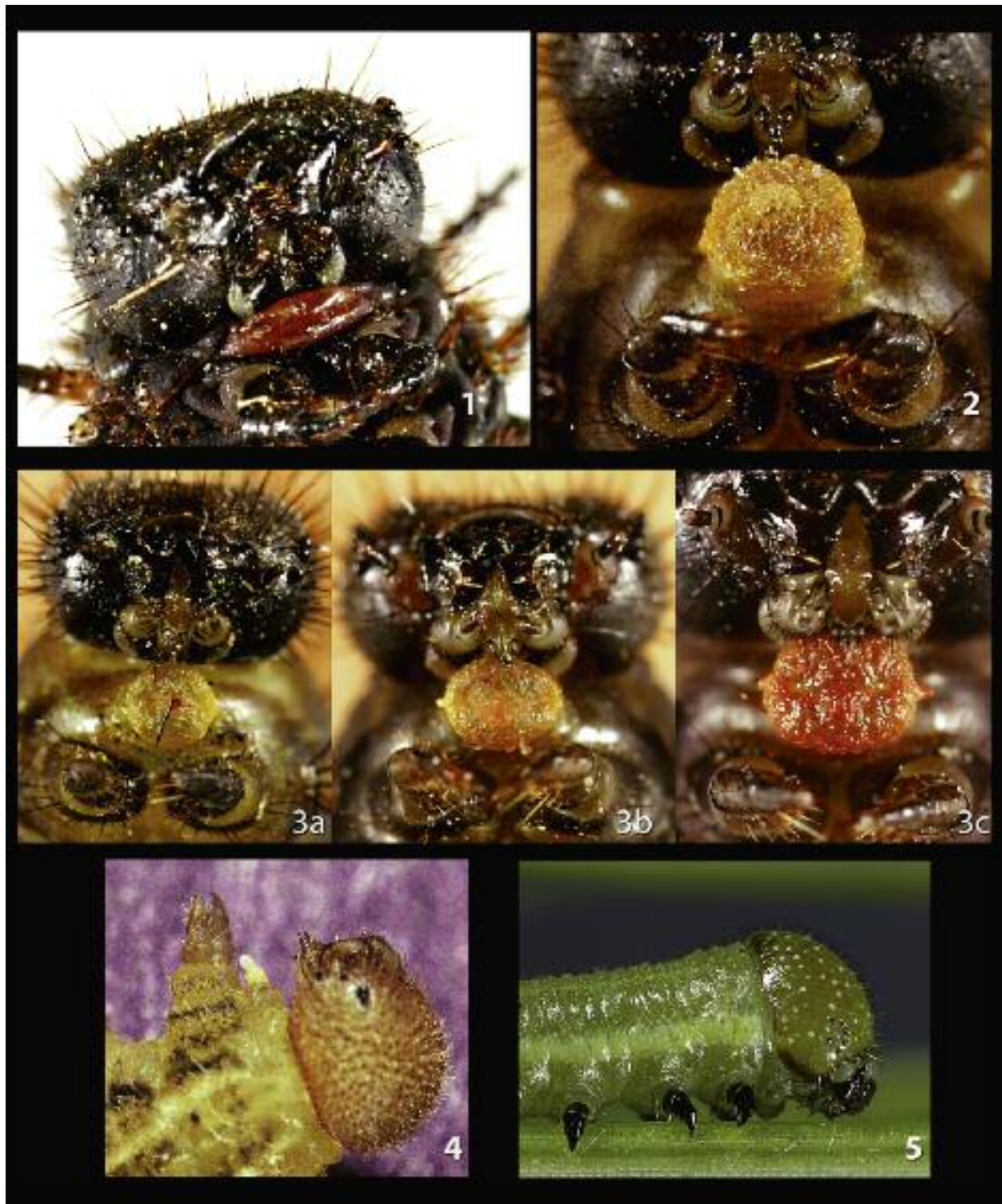
RESULTS

Observations on gland incidence and morphology. Larvae of at least 41 Pacific Northwest butterfly species belonging to three families (Nymphalidae, Hesperidae and Pieridae) possess a prosternal gland (Table 1). None of the larvae of Lycaenidae (23) and Papilionidae (8) had a prosternal gland. In the Pieridae, 11 species representing the genera *Neophasia*, *Pieris*, *Pontia*, *Euchloe*, *Anthocharis* and *Nathalis* were examined, and only *Neophasia menapia* and *Nathalis iole* Boisduval had larvae with a prosternal gland. Although only four species of Hesperidae were confirmed to have a prosternal gland, no other species were examined, but it is likely that larvae of all members of this family have a prosternal gland. Similarly, prosternal glands are likely to be a common feature of all Nymphalidae larvae, given that all nymphalid larvae examined in this study have a prosternal gland.

Observations on 7 *Argynnis* spp. used in chemical studies (below) showed the gland was not present in the first instar but appeared in the second instar as a pale-colored suture. In sixth instars, the non-everted gland was a transverse slit bordered by two 'lips' (Fig. 1). From the third to sixth instar, rough handling of larvae resulted in eversion of a single-lobed papilla (Fig. 2) and emission of a 'musky' odor. Gland size was comparable in all *Argynnis* spp. with the non-everted

TABLE 1. Pacific Northwest butterfly larvae confirmed to possess a prosternal gland.

Pieridae: <i>Neophasia menapia</i> , <i>Nathalis iole</i>
Nymphalidae: <i>Euptoieta claudia</i> , <i>Argynnis</i> (<i>Speyeria</i>) <i>cybele</i> , <i>Argynnis</i> (<i>S.</i>) <i>coronis</i> , <i>Argynnis</i> (<i>S.</i>) <i>zerene</i> , <i>Argynnis</i> (<i>S.</i>) <i>callippe</i> , <i>Argynnis</i> (<i>S.</i>) <i>egleis</i> , <i>Argynnis</i> (<i>S.</i>) <i>atlantis</i> , <i>Argynnis</i> (<i>S.</i>) <i>hesperis</i> , <i>Argynnis</i> (<i>S.</i>) <i>hydaspe</i> , <i>Argynnis</i> (<i>S.</i>) <i>mormonia</i> , <i>Boloria selene</i> , <i>Boloria bellona</i> , <i>Boloria epithore</i> , <i>Boloria freija</i> , <i>Boloria astarte</i> , <i>Boloria chariclea</i> , <i>Chlosyne acastus</i> , <i>Chlosyne palla</i> , <i>Phyciodes coccyta</i> , <i>Phyciodes pulchella</i> , <i>Phyciodes pallida</i> , <i>Phyciodes mylitta</i> , <i>Polygonia satyrus</i> , <i>Polygonia gracilis</i> , <i>Nymphalis l-album</i> , <i>Nymphalis californica</i> , <i>Nymphalis antiopa</i> , <i>Aglais milberti</i> , <i>Vanessa virginiensis</i> , <i>Vanessa annabella</i> , <i>Vanessa cardui</i> , <i>Vanessa atalanta</i> , <i>Junonia coenia</i> , <i>Adelpha californica</i> , <i>Erebia epipsodea</i>
Hesperidae: <i>Epargyreus clarus</i> , <i>Thorybes pylades</i> , <i>Pyrgus communis</i> , <i>Oarisma garita</i>



FIGS. 1–5. **1.** Non-everted prosternal gland of sixth instar *Argynnis coronis*. **2.** Everted prosternal gland of sixth instar *Argynnis hydaspe*. **3.** Everted prosternal gland in sixth instars of *Argynnis zerene* (**3a**), *A. egleis* (**3b**) and *A. coronis* (**3c**). **4.** Lateral view of everted prosternal gland in fourth instar of *Erebia epipsodea* (Nymphalidae: Satyrinae). **5.** Lateral view of prosternal gland in third instar *Neophasia menapia* (Pieridae).

NOTE: THIS NEEDS TO REFER TO FIGS. 3a, b and c rather than upper middle lower as stated in Word.doc fig. captions... check for consistency in text and caption for how images in plate are referred to. Please have author check to see labels & images for 3a,b,c are correctly matched.

gland in sixth instar *A. (S.) cybele* measuring 1.1–1.5 mm along the suture and the everted gland measuring 1.0–1.25 mm in width. Amongst *Argynnis* spp. there was a gradient in color of the everted gland from yellow-orange-red with *A. (S.) zereene* yellow, *(S.) egleis*, orange and *A. (S.) coronis* red (Fig. 3).

The prosternal glands of most other nymphalid (and hesperiid) species were similar in structure and size (although varied in color) to those found in *Argynnis* spp., with eversion and odor emission occurring when larvae were roughly handled. However, the gland in the only species examined in the subfamily Satyrinae was ovoid and the everted papilla was small and pale colored (Fig. 4). In the pierids *N. menapia*, and *N. iole* there were two bi-lobed glands situated laterally on the same segment (Fig. 5). In *N. menapia*, the glands appeared largest in the third and fourth instar, diminishing in the final (fifth) instar.

Chemical analyses: *Argynnis* spp. Chemical analyses revealed that prosternal gland extracts of final instars of seven *Argynnis* spp. contained hydrocarbons, fatty alcohols, carboxylic acids and acetate esters (Table 2). There were no apparent species-specific differences and data were combined. Extracts from all larvae examined (76) contained large quantities of dodecene or dodecanol and/or associated alkanes and acetate esters. Terpenoid compounds (e.g. germacrene-B, pentanoic acid, hexanedioic acid, 1, 3-bis (1, 1-dimethylethyl) benzene, 2, 6-dimethyl, 2, 6-octadiene-1, 8-diol) occurred in gland extracts of 61.8 % (47/76) of larvae. Squalene, a C30 polyunsaturated triterpene hydrocarbon was found in gland extracts of 19 larvae (25 %) and the secondary amines diphenylamine or diphenyl ether were found in gland extracts of 16 larvae (21 %). The alkaloid, 9, 10-dihydro-9, 9-dimethyl-acridine was found in the gland extracts of 17 larvae (22.4 %) and disulfide compounds were present in 23 extracts (30.3 %).

***Neophasia menapia* and *Polites sonora*.** The single extract samples from each of these species

showed similar chemistry to *Argynnis* spp., with dodecene/dodecanol and esters, and 1, 3-bis (1, 1-dimethylethyl) benzene present in each gland. The gland of *P. sonora* also contained diphenyl ether and diphenylamine with the latter also present in the gland of *N. menapia*.

DISCUSSION

Larvae of forty-one species of Pacific Northwest butterflies representing three families (Pieridae, Nymphalidae and Hesperidae) were found to possess a prosternal gland. Among 11 species of Pieridae examined only *Neophasia menapia* and *Nathalis iole* have this gland but all of the nymphalid species examined possessed it as did the four hesperids examined. It is likely that most if not all species in these two latter families have larvae with prosternal glands. In contrast, examination of lycaenid and papilionid larvae showed no evidence of prosternal glands. However, papilionid larvae are well documented to possess an analogous bifurcate eversible dorsal gland just behind the head, which secretes defensive chemicals (Eisner & Meinwald 1965; Omura et al. 2006).

Prosternal glands of *Argynnis* spp. larvae were very similar in size and form and varied in color from red to yellow. The glands of other nymphalid species also had similar morphology and were variable in coloration. The gland of the only species examined from the subfamily Satyrinae (*E. epipsodea*) was small and pale. Prosternal glands of hesperiid species ranged from red to brown and were similar morphologically to nymphalid glands. The prosternal glands of the pierid larvae, *N. menapia* and *N. iole*, differ significantly from the other families, in that both species have a pair of bi-lobed glands. In all species, the prosternal gland was absent in first instar larvae, appearing in the second instar and becoming progressively larger during development. An apparent exception occurred in *N. menapia* which had the glands largest in the third and

TABLE 2. Presence of compounds in prosternal gland extracts of *Argynnis* spp. (Nymphalidae) obtained and analyzed during 2007–09.

YEAR and # of extracts	dodecene dodecanol & esters	terpenes	squalene	diphenylamine diphenyl ether	acridine	disulfides
2007 (27)	X	X	X		X	
2008 (37)	X	X				X
2009 (12)	X	X		X		X

fourth instars, but smaller in the final instar. Rough handling of larvae usually resulted in gland eversion and, in *Argynnis* spp., emission of a noticeable musky odor.

Our analyses of prosternal gland emission chemistry in seven species of *Argynnis* showed the presence of dodecene or dodecanol and/or associated alkanes and acetate esters in nearly all samples. These compounds were also present in gland emissions of *N. menapia* and *P. sonora*. Dodecanol and associated acetate esters were found in the prosternal gland of mature *Datana ministra* (Drury) (Notodontidae) larvae and were suspected of acting as a 'dispersal pheromone' keeping the larvae solitary (Weatherston et al. 1986). Similar compounds, decyl acetate and dodecyl acetate, comprise the alarm pheromone of western flower thrips (*Thrips occidentalis* (Pergande)), which causes dispersion of conspecifics (Teerling et al. 1993). Dodecanol and acetate esters in prosternal gland secretions of *Argynnis* spp. caterpillars might serve to keep individuals well separated to reduce competition for host plant resources. Alternatively, the secretions may serve as a warning pheromone to disperse conspecifics when a larva is attacked by a predator.

Terpenoid compounds were found in most *Argynnis* spp. samples as well as in *N. menapia* and *P. sonora*, and likely have a defensive function. Terpenoids dominate the osmeterial secretions of early-mid instar papilionid larvae whose major enemies are invertebrate predators (Omura et al. 2006), and also were found in prosternal glands of nymphalid larvae, *Dione juno* and *Abanote hylonome* (Osborn & Jaffe 1998). All *Argynnis* spp. samples contained disulfides or squalene, but other potentially defensive compounds varied in their occurrence in the larvae examined. Acridine and/or diphenylamine were also found in some samples. Diphenyl ether and diphenylamine were found in *P. sonora*, but only the former was found in *N. menapia*. Squalene, a C30 polyunsaturated hydrocarbon and an intermediate in the biosynthesis of other triterpenoids and sesquiterpenoids (Bonner 1965), is the dominant component of the defense secretion of the American dog tick, *Dermacentor variabilis* (Say) and is repellent to fire ants, *Solenopsis invicta* Burren (Yoder & Domingus 2003). If squalene is repellent to ants generally, the value of this secretion to near ground or ground-dwelling caterpillars, such as those of *Argynnis* spp. and *Polites* spp., is apparent. Disulfides are generally toxic or repellent to insects (Huang et al. 2000) as are diphenylamine and diphenyl ether (Debboun et al. 2006). Despite some inconsistency in the GC-MS data apparent between the sampling years (which may have accrued from minor variations in

larval rearing methodology and GC-MS procedures), there was overall consistency in *Argynnis* spp. prosternal gland emissions which contained compounds with likely dispersive and defensive functions. However, more research is needed to demonstrate their functions experimentally.

The results of this study suggest that the prosternal gland occurs in larvae of most if not all species of the butterfly families Nymphalidae and Hesperidae. It is present also in larvae of at least two species of Pieridae. Based on our preliminary chemical evidence, the prosternal gland appears to have at least two functions: to defend and to disperse. Previous studies on the chemistry of prosternal gland emissions in notodontid and noctuid moth larvae also suggested defense and dispersal functions. The glands of *Schizura concinna* (J.E. Smith) and *Datana ministra* (Drury) larvae contain dodecyl acetate and/or dodecanol/dodecyl acetate, but only *D. ministra* also has a defensive compound (formic acid) (Weatherston et al. 1979, 1986). The prosternal gland of the noctuid, *Spodoptera frugiperda* contains saturated hydrocarbons, primarily *n*-pentadecane, likely to have a defensive function. The only previous chemical analysis of prosternal glands in butterflies was reported by Osborn and Jaffe (1998), who showed carboxylic acids and terpenes were present in prosternal gland secretions of the nymphalids *D. juno* and *A. hylonome*. These compounds were shown to be repellent to ants (Osborn & Jaffe 1998), thus the gland in these nymphalids appears to have a defensive function only.

The existence of a prosternal gland in larvae of two pierid species is noteworthy because most species in this family in the Pacific Northwest do not have this gland. Chemistry of the *N. menapia* gland suggests dispersive and defensive functions. Larval chemical defense in many pierid species is attributable to oily droplets attached to the tips of dorsal setae. Smedley et al. (2002) showed that in *Pieris rapae* (L.) these droplets contain unsaturated lipids (mayolenes) that repel ants. *Neophasia menapia* larvae do not carry oily droplets on their setae (James & Nunnallee 2011) and may rely instead upon the prosternal gland for defense. *Nathalis iole*, on the other hand, has setal droplets in instars 1–3 but no droplets and a well developed prosternal gland in the fourth (final) instar (James & Nunnallee 2011). *Nathalis iole* was the only species in the pierid subfamily Coliadinae that we examined. Other species in this subfamily may also possess prosternal glands.

The apparent widespread occurrence of prosternal glands in butterfly larvae of the families Nymphalidae and Hesperidae suggests that this gland is important in

the ecology of many species. Defense is likely to be a major function providing protection perhaps against ground-dwelling predators like ants, beetles and scorpions. Our study involved butterfly species in the temperate Pacific Northwest. Other studies have indicated some tropical butterfly larvae (Nymphalidae) also possess prosternal glands (Muysshondt & Muysshondt 1976; Aeillo & Silberglied 1978; Osborn et al. 1999; Borges et al. 2010). It would be useful to determine the relative incidence of prosternal glands among larvae of tropical and temperate butterfly species.

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