

Polistes smithii vs. *Polistes dominula*: the contrasting endocrinology and epicuticular signaling of sympatric paper wasps in the field

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Abstract Paper wasps of the family Vespidae exhibit a wide range of social lifestyles, from facultative eusocial groups to highly ritualistic swarm-founding societies. Even so, adult caste flexibility is widespread throughout the eusocial tribes. Thus, a common endocrine mechanism for caste determination and maintenance in paper wasps is expected, with *Polistes dominula* serving as a model for the study of mechanisms controlling phenotypic plasticity. In *P. dominula*, juvenile hormone (JH) and ecdysteroids have been shown to have important caste-determining functions, are important for reproductive growth, and correlate with hydrocarbon signals on the cuticle. Yet research on swarm-founding vespids has shown that JH functions are surprisingly labile, begging the question as to how conserved JH functions are within *Polistes*, a non-swarmling genus. Here, we compared the JH and ecdysteroid titers, cuticular hydrocarbon (CHC) profiles, and prospective visual signals of dominance of free-living foundresses from two sympatric and closely related species of *Polistes* in South Africa, the indigenous *Polistes smithii*

and the invasive *P. dominula*. In contrast to *P. dominula*, neither the JH titer nor the CHC profile was linked to dominance or reproduction in *P. smithii*, and in both species, hemolymph ecdysteroids were essentially absent. Moreover, many of the relationships between hormones, reproduction, dominance, and social signals in *P. dominula* are in contrast to studies performed on northern hemisphere populations. The divergence of endocrine and chemical profiles within *Polistes* offers an unforeseen opportunity to study the evolution of proximate mechanisms underlying phenotypic plasticity.

Keywords Cuticular hydrocarbons · Dominance · Ecdysteroids · Juvenile hormone · *Polistes* · Reproduction

Introduction

In most social animals, reproduction is not shared equally between members of the group, a result of competition between potential breeders over resources. In many groups, the most fertile individuals maintain their position through acts of dominance and/or pheromone signals which have the effect of suppressing the reproductive development of others. In small societies, all members have the potential to directly reproduce, yet reproduction may be limited to one individual which, through signals of dominance, effectively neuter subordinates that stay within the group to cooperatively raise the young (West-Eberhard 1996). In such groups, the reproductive prospects of an individual are affected by both condition- and context-dependent factors which, through endogenous signals, give rise to the expression of a distinct complex of behavioral and physiological traits. A major conceptual issue in evolutionary endocrinology is how hormone-mediated functions arise, and the extent to which they are constrained (Zera 2006; Hartfelder and Emlen 2012), a challenge which can be

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investigated both by the physiological interrogation of model organisms and by comparing the endocrine profiles of closely related species.

Paper wasps (i.e., the eusocial Vespidae) are an ideal group to study evolutionary endocrinology in social animals (Jandt et al. 2013). As with most non-swarving paper wasps, a *Polistes* society is characterized by a linear dominance hierarchy comprised of caste-flexible females (West-Eberhard 1969). Yet there is considerable variation in social behavior within and between species of *Polistes* (e.g., the propensity for foundresses to form groups or not (Liebert et al. 2008; Jandt et al. 2013) and ritualized signals (West-Eberhard 1982)).

In the well-studied temperate zone species *Polistes dominula*, potential reproductives emerge from diapause in the spring and associate at future nesting sites (i.e., foundresses), although some females may establish a nest on their own. On multiple-female nests, springtime foundresses compete to become the dominant reproductive (for the most recent review, see Jandt et al. 2013). Foundresses advertise condition-dependent visual signals of quality on their faces (Tibbetts and Dale 2004), employ physical and ritualized forms of aggression (Reeve 1991; Röseler 1991), eat the eggs of others (Gervet 1964; Liebig et al. 2005), and produce chemical signals which convey information about dominance and/or fertility status (Sledge et al. 2001; Dapporto et al. 2007a, 2010; Izzo et al. 2010). Prior to the emergence of the first generation of progeny (the “pre-emergent” phase), a stable linear hierarchy is established whereby the highest ranking female monopolizes reproduction while her subordinates help raise the young (Pardi 1948; Röseler 1991; Spradbery 1991). Once workers emerge (“post-emergent” phase), subordinate foundresses usually leave the colony and the remaining alpha foundress, now the queen, continues to ensure her position of dominance (see Jandt et al. 2013). Through both hormone measurements and manipulations, juvenile hormone (JH) and to some extent ecdysteroids, major orchestrators of insect development (Riddiford 1994; Riddiford et al. 2001) and reproduction (Nijhout 1994; Wyatt and Davey 1996), have been shown to influence caste fate and drive caste physiology of *P. dominula* females in both the pre-emergent (foundress-based) (Röseler 1991) and post-emergent (queen-worker-based) colonies (see Jandt et al. 2013).

Quantification of JH levels (determined directly or indirectly) has indicated that the JH level is higher in dominant foundresses than subordinate ones, although the magnitude of the difference probably depends on the phase of the colony. For example, the relationship between JH titer and dominance was less pronounced between paired foundresses from new pre-emergent nests (1–2 brood cells) than among paired foundresses from established pre-emergent nests (10–15 brood cells) (Tibbetts et al. 2011a). In post-emergent colonies, queens of *P. dominula* and the Neotropical *Polistes canadensis* (the only other *Polistes* species to have its

hormones measured prior to this study) have higher JH titers than workers which, in general, have lower but variable JH titers, which may or may not correspond to the performance of specific age-related tasks (Giray et al. 2005; Tibbetts and Huang 2010). Higher JH titers are also associated with large-bodied foundresses that showcase high-quality condition-dependent visual face marks which are used by rivals to assess their chance of winning a fight (Tibbetts and Banan 2010). Although dominance does not always correlate with ovary size in competing springtime foundresses (Tibbetts et al. 2011a), JH levels and production do correlate with oocyte length and growth (Röseler et al. 1980; Izzo et al. 2010).

Elevated JH titers are also associated with chemical signatures of fertility in *P. dominula* (Izzo et al. 2010). The cuticular hydrocarbons (CHCs) are vital for communication in many eusocial insects, often conveying information that pertains to an individual’s physiological state (Richard and Hunt 2013). In *P. dominula*, the CHC profiles of springtime foundresses contain information relating to their ovary size (Izzo et al. 2010), but once the dominance hierarchy is established, they have been argued to function as dominance signals (Dapporto et al. 2007a). A change in the communicative function of a foundress’ CHC profile is conceivable (Dapporto et al. 2010), but it is also possible that, instead, the differences relate to geographical and/or climatic variation (northern USA vs. Italian populations), just as visual signals of agonistic ability do (Tibbetts et al. 2011c).

Thus, like in many other insects, JH functions pleiotropically in *P. dominula*, coordinating the expression of multiple behavioral and physiological traits which come to define incipient and actual dominant reproductives (West-Eberhard 1996; Izzo et al. 2010; Hartfelder and Emlen 2012). Hormone manipulation studies, using synthetic JH or methoprene, a stable JH mimic (JHM), confirm this: treatment of both foundresses and workers augments dominance behaviors and accelerates reproductive development (Röseler et al. 1984; Röseler 1991), but this effect was dependent on the condition of the foundress (Tibbetts and Izzo 2009; Tibbetts et al. 2011b) and the social context (Shorter and Tibbetts 2009; Tibbetts et al. 2011b). Differential responses to JHM treatments may reflect a female’s ability, based on her relative condition (e.g., body size and fat body size/reserves) and social situation (e.g., presence of queen and state of brood), to adaptively channel energy toward physiological or behavioral processes that give the female the best chance to reproduce, whether directly or through helping (West-Eberhard 1996; Tibbetts et al. 2011b).

In addition to JH effects on dominance, ecdysteroids have been shown to augment fighting ability in foundresses. In *P. dominula* females, ecdysteroids are produced in the ovaries and are released into the hemolymph, and are much higher in egg layers than in foundresses emerging from diapause (Strambi et al. 1977; Röseler et al. 1985). As with JH

treatments, injections of the ecdysteroid 20-hydroxyecdysone (20E) into foundresses having smaller corpora allata (CA) and oocytes than their paired opponent boosted their chances of becoming dominant by sevenfold, similar to results obtained from JH as well as JH+20E treatments (Röseler et al. 1984, 1985). Yet ecdysteroids are certainly not required for the expression of dominance behaviors, since ovariectomized foundresses—provided they have enlarged CA—are able to become behaviorally dominant and maintain the alpha position, even though subordinates are allowed to reproduce (e.g., the eggs are not eaten) (Röseler and Röseler 1989). Whether a lack of circulating ecdysteroids affects possible dominance signals in the CHCs of *P. dominula* is not known, but in terms of agonistic behavior, elevated hemolymph ecdysteroids could only augment but not determine dominance.

P. dominula has thus become an important invertebrate model species for understanding how hormones (and intraspecific variation in hormone responsiveness) differentially regulate alternative phenotypes in primitively eusocial animals (Jandt et al. 2013; Tibbetts and Crocker 2014). Of equal interest are interspecific differences in the endocrine regulation of caste plasticity, especially within and between closely related groups (Tibbetts and Sheehan 2012). For instance, studies on primitively eusocial Neotropical vespid wasps (*Synoeca surinama* and *Polybia micans*) which establish nests by swarming, and likely share an ancestor with *Polistes* that was caste-flexible (Noll and Wenzel 2008; Pickett and Carpenter 2010), show strikingly divergent endocrine profiles from each other as well as from those reported for *P. dominula* and *P. canadensis* (Kelstrup et al. 2014a, b). This led us to ask how labile or flexible JH and ecdysteroid functions may be within the genus *Polistes*. We therefore chose to study two sympatric, closely related species of *Polistes* (belonging to the same Eurasian-African subgroup *Polistes sensu stricto* (Bequaert 1918; Pickett et al. 2006; Santos et al. 2014)) in South Africa: an alien invasive *P. dominula* and the indigenous *Polistes smithii* Saussure (hereafter referred to as *P. smithii*). Foundresses from these species nest alone or in groups, although in Stellenbosch, Western Cape, solitary nest founding appears to be much more prevalent in *P. smithii* (shown here). By contrast, *P. dominula* were observed to form large foundress associations, a phenomenon of recently invaded areas (Liebert et al. 2008). The two species also have distinct biogeographic histories: whereas the ancestors of *P. dominula* evolved a diapause-based colony cycle in the northern hemisphere, *P. smithii* is adapted to the southern hemisphere. Therefore, these species evolved a diapause-based colony cycle independent from one another, likely from a common tropical ancestor (Santos et al. 2014), raising the possibility that independent adaptations to temperate environments may have involved different hormonal mechanisms. Relating to this, we were interested if *P. dominula*, a recent invader to South Africa (Benadé et al. 2014), has a

comparable endocrine profile and relationship to behavior and CHC signaling as in populations outside South Africa.

Based on studies of *P. dominula*, we chose to focus on late pre-emergent nests of *P. smithii* when endocrine titers and CHC profiles are expected to differ the most between female cohorts (due to divergence in fertility and social status). We expected, based on comparative studies within the genus (see Tibbetts and Sheehan 2011), that foundresses of Stellenbosch *P. dominula* would be headed by relatively large alpha females with correlated patterns of visual marks shown to signal dominance (Tibbetts and Lindsay 2008), while variation in facial marks would not be expected for sympatric *P. smithii* lone foundresses and small foundress groups. Given their close relatedness, their shared environment and comparable colony cycle, and the growing support for endocrine-mediated dominance in social Hymenoptera (Sledge et al. 2004; Tibbetts and Huang 2010; Smith et al. 2013; Kelstrup et al. 2014b), we hypothesize that JH and ecdysteroid titers of foundresses would show similar patterns in the two species, with alpha foundresses showing higher levels of both hormones than subordinate ones, and in the case of *P. smithii*, higher levels than lone foundresses. We were also interested whether these hormone correlate with the CHC profile of the two species and, furthermore, whether the CHC profile more strongly associates with fertility or social status, which for *P. dominula* is a contentious topic (Dapporto et al. 2010; Izzo et al. 2010). And although this study was set up to juxtapose two closely related species of *Polistes*, it also affords a comparison of the social biology of South African *P. dominula* to well-studied populations in the northern hemisphere.

Material and methods

Wasp colonies

Colonies of both species were studied in situ in Stellenbosch, South Africa, from early November to mid-December 2013. The field site for *P. smithii* included buildings in a 2-km² area on the south side of Jonkershoek road [Cape Nature offices at the Assegaaibosch (S 33° 58' 5", E 18° 15' 21"), Die Eiland Campsite (S 33° 57' 35", E 18° 55' 4"), and neighboring homes along Jonkershoek Road (33° 57' 28", E 18° 54' 45")]. Five to ten nests were monitored at each site. Eleven colonies of *P. dominula* were studied at Le Verger B&B on Devon Valley Road (33° 55' 1", E 18° 49' 0") along with one colony from Dornier Wine Estate (33° 59' 32", E 18° 52' 19"), located 5.5 km distant from the Le Verger site.

In both species, colonies were in the late pre-emergence stage, meaning that all females were foundresses on nests with pupae but no young adults yet. All observed females were marked with Sharpie paint pens on the thorax at least 3 days before observations began, and in nests with multiple

foundresses, behavioral observations were performed until the alpha and beta foundress statuses were clear (see below). Dominance behaviors included the mounting and/or biting of a nestmate, while subordinate behavior, in response, consisted of crouching and/or an offering of regurgitated liquid droplets (see Fig. 1 in Jandt et al. 2013). Foundresses were considered lone foundresses if seen to be alone at night (after 18:00) and if no other wasp was observed to visit the nest during the 2- to 3-day period prior to their removal. In nests with multiple foundresses, 3 days of observation (which included video recordings) was recorded at each site.

For each nest, a pairwise matrix of dominance-subordinate encounters was created. In smaller two-foundress nests, where the only dominance interactions consisted of one female dominating the other, the alpha was identified as the dominant one. For all other nests, we employed a Bayesian analysis for assessing the probabilities of dominance ranks within colonies with OpenBUGS 3.2.3 software (see Adams 2005) rather than using traditional non-parametric models for assessing dominance ranks. The Bayesian approach generates measures of certainty, called posterior probabilities, for each possible hierarchy, so that, for example, $A > B > C$ can have a probability of 0.8, $A > C > B$ a probability of 0.1, $B > A > C$ a probability of 0.05, and all other arrangements would then have a cumulative posterior probability of 0.05. In all *Polistes* colonies studied here, one female was consistently ranked as the most dominant one in the hierarchy, receiving a top-rank posterior probability score of 0.99 or greater, meaning the identification of the alpha foundress was clear-cut. Beta (i.e., second-ranked) foundresses were determined, unambiguously, by the same calculation.

For *P. smithii*, 28 nests were collected, which included 18 lone foundresses, 10 alpha foundresses, and 18 subordinate foundresses. Seven alphas shared their respective colony with a single subordinate, although only five of these subordinates could be collected for physiological measurements. The three other nests, including the alpha, contained three, four, and seven foundresses. An additional four females, which were not observed long enough to determine if they were lone foundresses or not, were removed from other nests. For *P. dominula*, we observed 12 multiple-foundress colonies, containing 2 ($n=4$), 3 or 4 ($n=4$), 6–8 ($n=2$), 12, and 19 females, the largest one located at Dornier Wine Estate. Although South African *P. dominula*, like other invasive populations, often reuse old nests from the year prior (PC Benadé, personal communication), all of the nests in this study appeared to be newly constructed (e.g., the areas studied were cleared of paper wasp nests before springtime, and no shriveled brood and meconia were observed inside collected nests; see Liebert et al. 2008).

In *P. dominula*, foundress body size and facial pattern variability are increased in locations with colder winters (Tibbetts et al. 2011c). In Stellenbosch, the overall average, average

high, and average low daily temperatures in June 2013, the coldest month of the year, were 12.0, 18.1, and 5.9 °C, respectively. At no point in 2013 did the temperature drop below zero (data obtained from the South African Weather Services weather station in the Assegaaibosch Nature Reserve); therefore, facial pattern variability was not likely to be temperature related.

Collecting hemolymph for hormone measurements, cuticular hydrocarbon wash, and ovary measurements

All wasps from a given site were removed on the same afternoon (13:00–17:00) and placed in clean glass vials and immediately buried in ice. The cold-immobilized (but not frozen) females were then bled within 2 h of collection since longer periods of cold anesthesia can significantly affect JH titers in honeybees (Lin et al. 2004). Two to 7 µL of hemolymph was withdrawn from between the anterior-most segments of the gaster with a microcapillary (Hirschmann ringcaps®, Eberstadt, Germany). Samples destined for JH and ecdysteroid measurements by radioimmunoassay (RIA) were transferred to glass vials containing 500 µL of acetonitrile (for JH) or 500 µL of methanol (for ecdysteroids).

Subsequent to bleeding, cuticular hydrocarbons (CHCs) were extracted from females by placing them in 1 mL of hexane ($\geq 99.9\%$) for 2 min. Hormone and CHC samples were kept at -20 °C, and the females were kept on ice and transported to the laboratory. In the evening of the same day, the individuals were placed in cold Ephrussi and Beadle Ringer solution (7.5 g NaCl and 0.35 g KCl/1 L distilled water) for ovary dissection. The ovaries were photographed with a Canon PowerShot G9 digital camera attached to a Zeiss Stemi DV4 microscope. The six longest oocytes were measured using ImageJ (NIH, Bethesda, MD, USA) and averaged. The ovaries of a subset of *P. dominula* were preserved in 100 % methanol for ecdysteroid measurements. The wasps were then preserved in 70 % ethanol. Later, the faces of these wasps were photographed using the same setup as above, and ImageJ was used to measure the widest part of the head. In *Polistes*, head width is considered a good indication of structural body size (Zanette and Field 2009; Tibbetts and Sheehan 2011).

Hemolymph juvenile hormone titer analysis by radioimmunoassay

The JH extraction and RIA procedure were the same as used in our previous JH titer analyses of the South American *S. surinama* and *P. micans* wasps (Kelstrup et al. 2014a, b). For the radioimmunoassay, we used [$^{10}\text{-}^3\text{H(N)}$]-JH III (specific activity 19.4 Ci/nmol, PerkinElmer Life Sciences, Waltham, MA, USA), JH-III (Fluka, Munich, Germany), and a JH-specific antiserum (Goodman et al. 1990). JH titers of the

samples were calculated by non-linear four-parameter regressions on standard curve values (ImmunoAssay Calculations spreadsheet, Bachem, Bubendorf, Switzerland) and are expressed as JH-III equivalents (pg/ μ L hemolymph).

Hemolymph ecdysteroid titer analysis by RIA

Hemolymph and ovary samples in 500 μ L in methanol were cold centrifuged (4 °C), and the supernatant was transferred to RIA glass vials and dried by vacuum centrifugation. Interfering lipid was removed from the ovarian extract in methanol as described by Geva et al. (2005) and modified by Kelstrup et al. (2014a). The RIA procedure is equivalent to the one used in our previous ecdysteroid level analyses of *S. surinama* and *P. micans* wasps (Kelstrup et al. 2014a, b). We used an antiserum prepared against a hemisuccinate derivative of ecdysone (Bollenbacher et al. 1983; Feldlaufer and Hartfelder 1997), [23,24-3H(N)]ecdysone (PerkinElmer, spec. act. 102 Ci/mmol), and 20-hydroxyecdysone (20E; Sigma, St. Louis, MO, USA). Results are expressed as 20E equivalents and were calculated by the same four-parameter regression analysis used for JH titers (see above).

Cuticular hydrocarbon analysis

The CHC extracts were concentrated to \sim 150 μ L under a stream of pure nitrogen. One microliter of the concentrated sample was injected into an Agilent 6850 gas chromatograph (GC) to quantify the hydrocarbon profiles of the wasps. The GC system was fitted with a splitless inlet, flame ionization detection, and a DB-5 capillary column (30 m \times 0.32 mm \times 0.25 μ m film thickness, Agilent Technologies, CA). The injection port and the detector were set at 290 and 320 °C. Helium acted as the carrier gas at a flow rate of 30.4 mL/min, with nitrogen acting as the make-up gas. The temperature was programmed as follows: 1 min at 150 °C, increased to 250 °C at a rate of increase of 15 °C/min, and then a further increase to 310 °C at a rate of increase of 3 °C/min, where the temperature was held for 30 min. Gas chromatograms were generated using GC ChemStation software (Rev. A.09.03, Agilent Technologies, 1990–2002).

Representative samples of each species were then analyzed by GC-coupled mass spectrometry (GC/MS) using an Agilent GC 6890N with 5975 MSD fitted with a ZB-5MS GUARDIAN (30 m, 0.25 mm ID, 0.25 μ m film thickness) ZB 7HG-G010-11 column. An authentic C₇–C₄₀ straight-chain hydrocarbon series (Supelco[®] Analytical, Bellefonte, USA) was used as a standard to identify n-alkanes and to link the retention times in our dataset. Non-linear alkanes were identified by analyzing the fragmentation patterns of the EI-MS and the presence of diagnostic ions (see Blomquist et al. 1987).

Size and facial pattern analysis

In *Polistes* wasps, body size and (if present) facial pattern “brokenness” have been described as good predictors of dominance (Tibbetts and Lindsay 2008). Wasp heads from both species were photographed with a ruler under a dissecting microscope. Using ImageJ, the widest part of the head was measured and used as an index of body size. For *P. dominula*, these same photographs were used in Adobe Photoshop (San Jose, CA, USA) to analyze facial pattern brokenness on the clypeus, following the method of Tibbetts et al. (2011c). In brief, we created a 30 \times 60-pixel bitmap of the clypeus containing the area where variability in pigment patterns is seen. From this bitmap, we counted the black pixels from each vertical column across the horizontal length of the bitmap (excluding the first and last five columns which may be black due to pigment at the edge of the clypeus). The standard deviation of the black pixel counts was used as an index of brokenness (Tibbetts et al. 2011c).

Statistical analysis

General linear mixed models (GLMMs) were used to test differences in head width, facial pattern brokenness, and JH titers between female statuses, using status (e.g., lone foundresses, alpha foundresses, and subordinate foundresses) as a fixed effect and colony as a random factor. To explicitly test for differences in the pattern of JH titers between the species, based on status, we assessed whether or not the interaction between status and species was significant. In some cases, the Hessian matrix was unable to compute the standard errors of the covariance parameters due to the covariance estimates being equal to 0, indicating that colony as a random factor caused non-significant variation in the data. Least square differences (LSD) between female statuses were used for post hoc pairwise comparisons. Furthermore, in *P. dominula*, the JH titers of alphas and fecund betas were directly tested with a matched pair analysis. Data for facial brokenness was log transformed to improve normality and to afford a direct comparison with other populations of *P. dominula* which used Pearson’s correlation (Tibbetts et al. 2011c). For both *P. smithii* and *P. dominula*, the average length of the six longest oocytes (average oocyte, viz. egg chamber length, AOL), a measure of ovary size used for both species, showed a binomial distribution, with nearly all of the females possessing undeveloped and/or well-developed oocytes. For example, in *P. dominula*, all females except for two had an AOL of <0.75 mm or >1.5 mm (AOL range=0.2 to 2.94 mm), whereas all but four females of *P. smithii*, a smaller species, had an AOL of <0.5 mm or >1 mm (AOL range=0.2 to 1.93 mm). We refer to these foundresses with small and large AOL as having low and high fertility potential, respectively.

As log transforming oocyte data to improve normality failed, the non-parametric Kruskal-Wallis ANOVA was used to test for differences according to status, and the Mann-Whitney *U* test and Steel-Dwass method (a non-parametric version of the Tukey HSD) were used for single and multiple pairwise comparisons, respectively. All above statistical analyses were done in JMP 11.1 (SAS Corporation, Cary, NC, USA) and SPSS 22 (IBM, Armonk, NY, USA).

The CHCs were quantified by determining the peak area in the chromatograms. Foundresses were categorized based on social status or fertility class (low vs. high). Peaks which appeared in <30 % of the samples and were not associated with status or fertility potential were excluded from the analysis. We excluded small peaks (< 0.1 %) only if they were unidentifiable as hydrocarbons and were not associated with female status. In cases where a peak was undetectable, we assigned a proportional area percentage of 0.001 %. The compound percentage for each sample was then adjusted to 100 %, and all percentages were subjected to log ratio transformation (Aitchison 1982). To discriminate the CHC profiles of foundresses according to status or fertility potential, we performed principal coordinate analyses (PCoA) using a PerMANOVA test in R software (version 3.1.1; package *vegan*: version 2.0-10). Following the example of Falcón et al. (2014), we included 10,000 permutations. [To ensure that we did not overlook small qualitative differences between *P. smithii* females of different statuses, we superimposed GC chromatograms of alpha foundresses with those of their non-fertile subordinates ($n=7$ pairings). No regular discrepancy in peak representation between the foundress statuses was found.] Finally, using R integrated with Statistica 12.0 (StatSoft, Tulsa, OK, USA), multiple factorial analyses were performed for each species, whereby two principal components (or dimensions) were derived similar to that of a standard PCA analysis. Correlations between the variables (hydrocarbon proportions, JH titer, and AOL) were plotted against the first two principal components on a correlation circle graph where strong correlations (Pearson's $r>0.7$) appear in the outer circle (Abdi et al. 2013).

Results

Morphological measurements

P. smithii

Alpha females did not have wider heads than their subordinates (GLMM: $F_{1,15,7}=1.40$, $p=0.25$, $n=8$ alphas and 14 subordinates; colony did not have an effect). To test the possibility that foundresses use visual patterns on the face to signal dominance or identity, we ordered 16 foundress wasp heads (from eight nests, including that of the alpha along with a smaller

headed subordinate, if present) from the widest to narrowest and looked for differences in visual features (Figure S1). Slight variation in yellow markings in the lower clypeus are evident, but the pattern was not nearly as variable as that seen in *Polistes* species that receive information from facial markings (Tibbetts and Sheehan 2011, and references therein).

P. dominula

Alphas had wider heads than their subordinates (GLMM: $F_{1,62,1}=16.9$, $p<0.005$, $n=12$ alphas and 56 subordinates; colony had no effect) but did not have a higher facial pattern brokenness (GLMM: $F_{1,59,6}=0.187$, $p=0.667$). Four of the 12 alphas had little or no black markings on their clypeus, and overall, head width showed no association with facial pattern brokenness (Pearson's $r=-0.07$, $p=0.55$, $n=68$) (Figure S2). The mean head width was 3.54 mm (± 0.02 SE) and the mean score for facial brokenness was 3.75 (± 0.32 SE), both values being intermediate between Italian and northern USA populations of *P. dominula* (Tibbetts et al. 2011c).

Social status and oocyte length

P. smithii

Alpha and lone foundresses had relatively large oocytes while subordinate foundresses showed a great range in average oocyte length (AOL) (Fig. 1a). On two-foundress nests, the alpha foundresses had large egg-possessing ovaries (AOL median=1.46 mm, 95 % CI [1.23, 1.67], $n=7$), whereas the subordinates had much reduced, borderline filamentous ovaries (AOL median=0.26 mm, 95 % CI [0.17, 0.36], $n=5$) (Mann-Whitney *U* test: $Z=2.76$, $p=0.006$). AOL of the lone foundresses (AOL median=1.36 mm, 95 % CI [1.30, 1.54], $n=18$) was similar to that of alphas from two-foundress colonies. In nests with more than two foundresses (indicated by open circles in Fig. 1a), the alpha foundresses had especially long oocytes (AOL median=1.89 mm, 95 % CI [1.67, 2.06], $n=3$), and two of ten subordinate foundresses on these nests had an AOL exceeding 1.75 mm (AOL median for all ten subordinates=0.82 mm, 95 % CI [0.54, 1.34]) (Fig. 1a), indicating the presence of reproductive competitors.

P. dominula

All but one alpha foundress had well-developed ovaries (Fig. 2a). Alphas had longer oocytes than subordinates (Mann-Whitney *U* test: $Z=3.50$, $p=0.0005$), and the latter largely fell into two classes, with 28 subordinate foundresses possessing small, regressed ovaries (AOL <0.75 mm, considered to have “low fertility potential”),

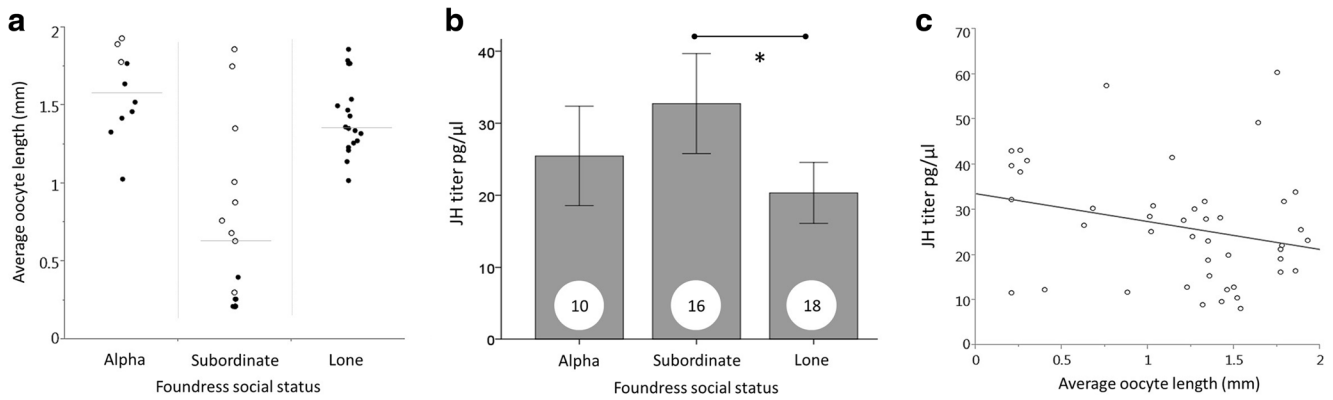


Fig. 1 Oocyte length and JH titers in foundress of *Polistes smithii*. **a** Alpha foundresses and lone foundresses had long oocytes compared to subordinate ones. Females taken from colonies with more than two foundresses are represented by open circles. Gray lines indicate the median lengths for each foundress type. **b** JH titers (mean±SD) of

foundress types. Only subordinate and lone foundress were significantly different (GLMM→LSD: $*=p=0.003$). Samples sizes are indicated in white circles. **c** JH titer was not significantly associated with oocyte length, especially so when only alpha and subordinate females were taken into consideration (for statistics, see text)

26 subordinates having relatively long oocytes (AOL range=1.6 mm–2.59, “high fertility potential”), and only 2 subordinates with an AOL between these lengths. As with *P. smithii*, subordinates on two-foundress nests had low fertility potential ($n=4$ colonies), whereas subordinate foundresses with high fertility potential were present in seven of eight colonies with more than two females. The presence of subordinate foundresses with high fertility potential, with oocyte lengths not significantly different from those of alphas (Kruskal-Wallis test: $H=2.09$, d.f.=1, $p=0.15$, $n=38$; see Fig. 2a), allowed us to test the whether social status, separated from oocyte length, had an effect on the endocrinology and chemical profile of foundresses. Therefore, for some analyses, subordinate foundresses with high fertility potential were split into

two groups based on their observed social status: beta (second-ranked) foundresses with high fertility potential and lower-ranking subordinates with high fertility potential.

Hormone measurements

P. smithii

Alpha females from nests with more than two foundresses ($n=3$) – where egg-possessing subordinates were present (see above) – had JH titers below and within 5 pg/μL of the mean of alphas from two-foundress nests ($n=8$). Ecdysteroid titers were very low (mean=0.92 pg/μL; SD=2.2; max=11.7 pg/

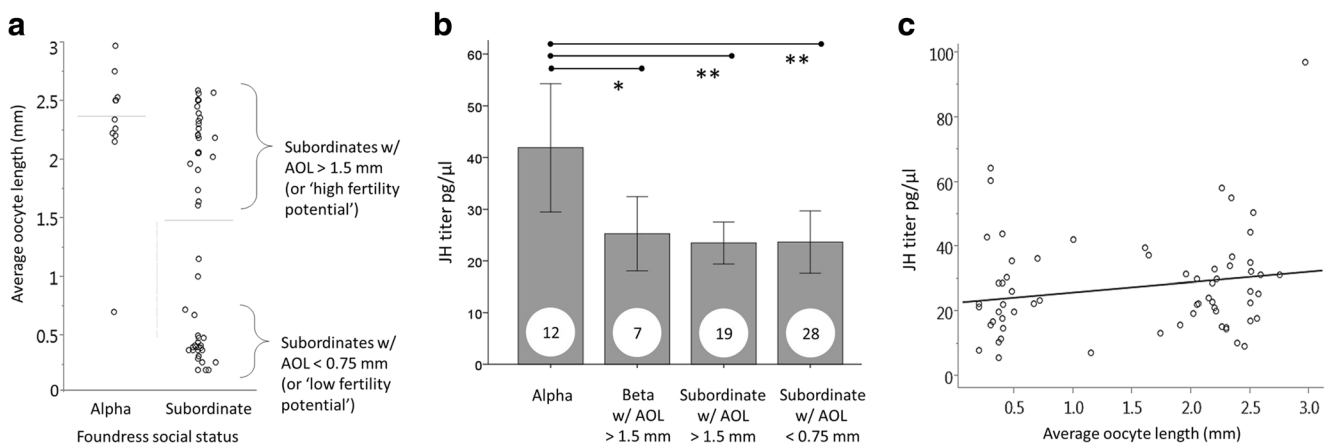


Fig. 2 Oocyte length and JH titers in foundress of *Polistes dominula*. **a** All but one alpha foundresses had well developed oocytes, whereas subordinates had a range of average oocyte lengths (AOL), with all but two foundresses having an AOL <0.75 or >1.5 mm. Gray lines indicate the median AOL for each foundress type. **b** Alphas had higher JH titers than subordinates, whether they were beta females with an AOL

>1.5 mm, lower-ranking foundresses with an AOL >1.5 mm, or subordinates in general with an AOL <0.75 mm (GLMM→LSD: $*=0.023$; $**=0.001$). Samples sizes are indicated in white circles. **c** JH titer did not associate with oocyte length ($n=64$, Spearman $\rho=0.22$, $p=0.146$)

μL , $n=44$) and showed no relationship with AOL (Spearman $\rho=0.003$, $p=0.99$).

JH titers showed a significant difference according to foundress status (GLMM: $F_{2,42}=4.94$, $p=0.012$), but surprisingly, among pairwise comparisons, only subordinate foundresses had higher JH titers than lone foundresses (Fig. 1b). Indeed, JH titers were, if anything, negatively associated with AOL (Spearman $\rho=-0.281$, $p=0.056$) (Fig. 1c), when all three groups were considered (alphas, subordinates, and lone foundresses). When considering the two groups of females from multiple-foundress colonies only (alpha foundresses and subordinates), which are the two groups directly comparable to the *P. dominula* nests, there was no relationship (Spearman $\rho=-0.231$, $p>0.2$).

P. dominula

Alpha females had higher JH titers than subordinates in general (GLMM: $F_{1,62}=13.94$; $p<0.0005$), as well as subordinates split according to their fertility potential and social rank (GLMM: $F_{3,60}=4.59$, $p=0.006$; Fig. 2b). Even if the abnormally large 19-foundress colony was removed from the model, a significant difference was maintained (GLMM: $F_{3,41}=3.55$, $p=0.02$), with alphas having significantly more JH than all subordinates (LSD: $p<0.05$) but producing similar JH titers to the betas with high fertility potential (LSD: $p=0.06$). Yet a more direct test for difference in JH titers between alphas and fecund betas, a matched pair analysis, showed that alphas indeed had higher JH, with a statistically significant mean difference of 25.03 pg/ μL (95 % CI [7.3, 42.7]; $t(6)=3.46$, $p<0.013$) with all colonies included and a statistical significant mean difference of 25.5 (95 % CI 3.6 to 47.5 pg/ μL ; $t(5)=3.0$, $p<0.03$) with the largest colony excluded. Among alphas, JH was higher in those with more subordinates in the colony ($\rho=0.58$, $p=0.046$), a pattern not seen in *P. smithii* (see above). Indeed, when comparing interspecifically the pattern of JH titers in alphas vs. subordinate foundresses (from all colonies), the interaction effect of status and species was significant, indicating that JH titers varied significantly based on the species and the status of the wasps (GLMM: $F_{3,83}=5.1$, $p=0.003$). However, JH titers did not correlate with AOL (Spearman $\rho=0.22$, $p=0.146$, $n=64$) (Fig. 2c).

As with *P. smithii*, ecdysteroid titers were very low (mean = 0.43 pg/ μL ; SD = 1.27; max = 8.54 pg/ μL , $n=65$) and did not show a relationship with AOL (Spearman $\rho=0.048$, $p=0.71$). This is not for lack of ecdysteroid content in the ovaries, which showed a significant correlation with oocyte length (Spearman $\rho=0.74$, $p<0.0001$, $n=53$) (see Figure S1). In fact, it appears as if there are two groups for oocytes >2 mm: one with less and the other with more than 75 pg. Nonetheless, this apparent split in high ecdysteroid content variation in ovaries with AOL >2 mm is not associated with alpha vs. subordinate females.

Cuticular hydrocarbon profiles

P. smithii

A total of 54 hydrocarbons were identified. The cuticular hydrocarbon (CHC) profile included linear alkanes (8.08 %), monomethyl-alkanes (48.39 %), dimethyl-alkanes (42.46 %), trimethyl-alkanes (0.51 %), and alkenes (0.47 %). The average CHC profiles of foundresses with low and high potential fertility (AOL <0.5 and >1 mm, respectively) are shown in Fig. 3a, whereas average percentages according to social status are indicated in Table S1. Of the 54 hydrocarbons, 11 hydrocarbons showed a significant correlation with AOL, and 9 of these relationships were negative (Fig. 3a).

According to the PerMANOVA, there was no significant difference in the CHC profiles of foundresses with large (>1 mm AOL; $n=32$) vs. small oocytes (<0.5 mm AOL, $n=9$) (Pseudo- $F=0.43$, $p=0.52$). Since lone foundresses would not necessarily be expected to produce a signal advertising fertility and/or dominance, we performed the same analysis, based on oocyte length, which included only social foundresses, and again no difference was found ($n=19$; Pseudo- $F=1.53$, $p=0.23$) (Fig. 4a). Indeed, the social status of a foundress did not seem to influence the CHC profile, as lone ($n=18$), alpha ($n=9$), and subordinate ($n=16$) foundresses showed no difference (Pseudo- $F=0.86$, $p=0.44$) (Fig. 4b). Therefore, neither foundress status nor ovary condition appears to be associated with quantitative differences in the CHC profile of *P. smithii*.

JH titer correlated with only 1 of the 54 hydrocarbon peaks (Fig. 3a). The overall lack of an association between JH titer, AOL, and the 54 hydrocarbon proportions (of total composition) is corroborated by a multiple factor analysis, in which these variables are plotted in correlation circle graphs against the first two dimensions (Fig. 6a). Neither AOL nor JH showed a strong association with the general directionality of the hydrocarbon proportions, indicating weak correlation.

P. dominula

A total of 71 hydrocarbon peaks were identified. The CHC profile consisted of linear alkanes (28.09 %), monomethyl-alkanes (32.97 %), dimethyl-alkanes (20.73 %), trimethyl-alkanes (0.73 %), and alkenes (17.48 %). In contrast to *P. smithii*, the CHC profile of *P. dominula* showed obvious fertility-associated differences (Fig. 3b). Fifty-one of the 71 hydrocarbons showed a significant correlation with AOL, including 11 of the 12 hydrocarbons which contributed more than 2.5 % toward the overall variance in the CHC profiles. The proportional representation of 6 of these 11 hydrocarbons showed a highly significant correlation with AOL and is shown in Fig. 5. This includes two highly correlated alkenes,

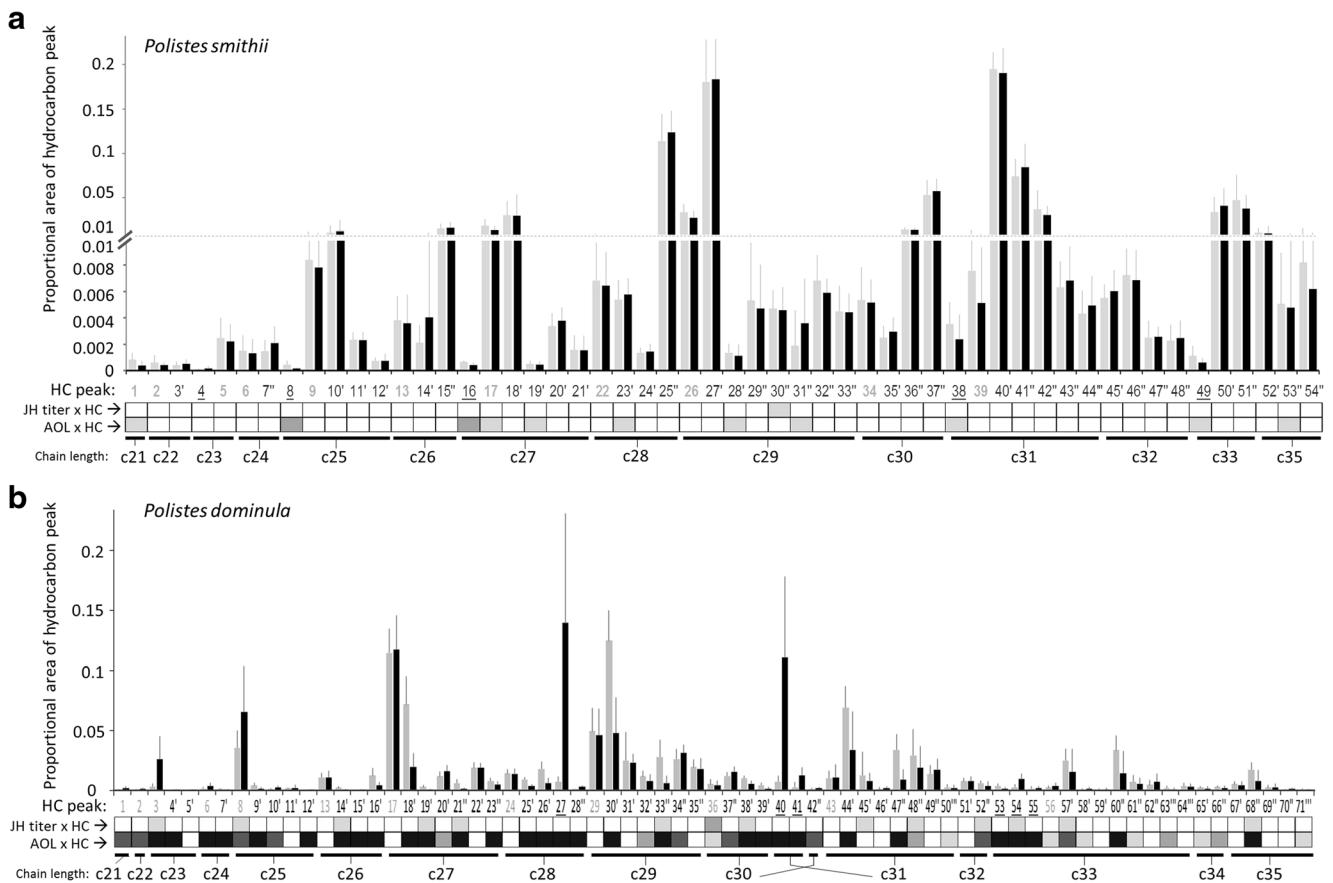


Fig. 3 **a, b** Proportional representation of cuticular hydrocarbon compounds (CHCs) for *P. smithii* and *P. dominula*. In both graphs, females with relatively small oocytes (<0.5 mm for *P. smithii* and <0.75 mm average length) (gray bars) are compared to females with relatively large oocytes (>1.0 mm *P. smithii* and >1.5 mm for *P. dominula*) (black bars). Error bars indicate standard deviation. Hydrocarbon (HC) identification number is shown (refer to Tables S1 and S2), and the font with symbols of the identification number indicate the type of hydrocarbon: *Light gray numbers* indicate linear alkanes and *underlined numbers* indicate alkenes. Methyl-branched CHCs are

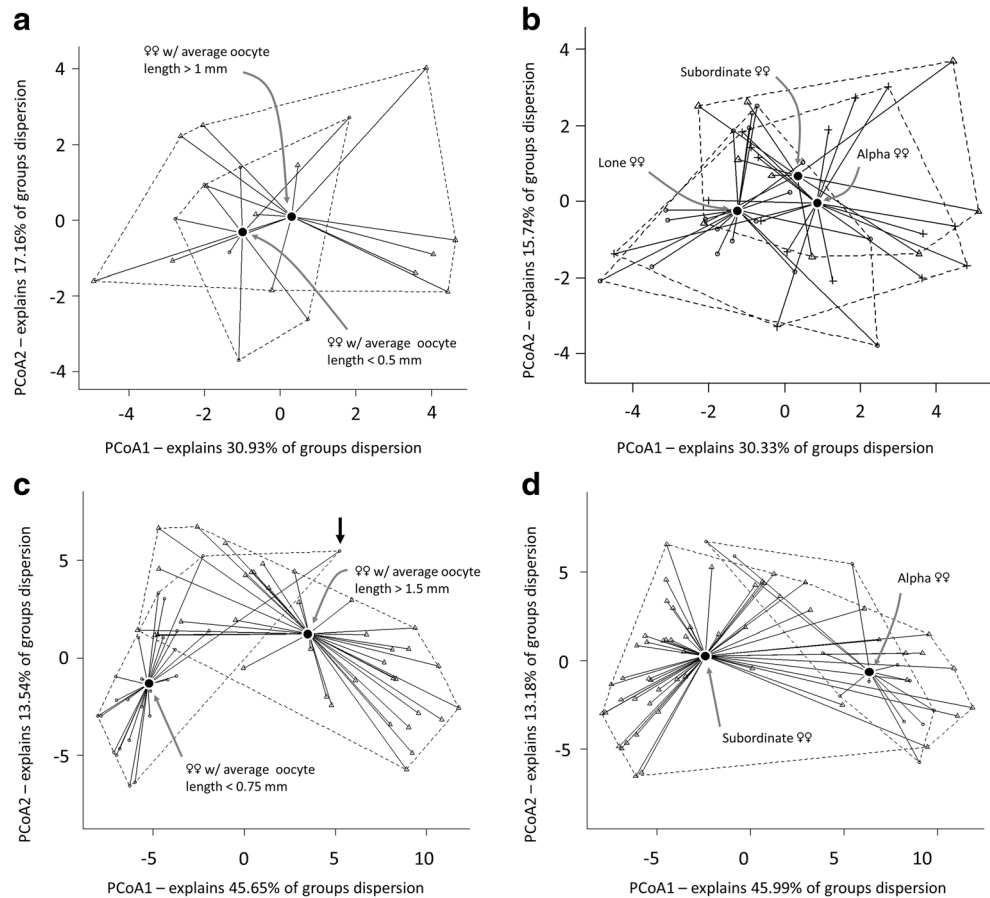
indicated with an *apostrophe* ('), and the number of apostrophes signifies the number of methyl branches. The chain length of the hydrocarbons is also indicated. As an example, 29'' for *P. smithii* represents a dimethyl-C29 hydrocarbon. The *two boxes* below each bar graph indicate significant correlations between either juvenile hormone titer (JH) or average oocyte length (AOL) and the corresponding hydrocarbon proportion. *Boxes filled in light gray, dark gray, and black* indicate correlations with p -values of <math><0.05</math>, <math><0.001</math> and <math><0.0001</math>, respectively

Z-C29 and Z-C31a, which were, with some exceptions, substantially higher in females with developed ovaries (Fig. 3b). There was a significant difference in the CHC profiles of foundresses with large oocytes (>1.5 mm AOL; $n=37$) compared to foundresses with small oocytes (<0.75 mm AOL, $n=27$) (Pseudo-F=11.28, $p=0.002$) (Fig. 4c). Alpha foundresses ($n=12$) did not differ from subordinates ($n=54$) (Pseudo-F=0.67, $p=0.43$) (Fig. 4d; see Table S2 for percent representation of each CHC), and there was extensive overlap in group dispersion between alphas, betas, and lower-ranking foundresses with developed ovaries (Pseudo-F=0.69, $p=0.50$) (Figure S3). These results show that oocyte length (fertility potential) is a better overall predictor of the CHC profile than social status. Yet it is also true that an alpha female with very small oocytes had a CHC profile which resembled that of fertile foundresses (Fig. 4c, black arrow), and some low-ranking foundresses with large oocytes intermixed with

foundresses with small oocytes (Fig. 4c), showing that fertility—as quantified by average oocyte length—is not an absolute predictor of the CHC profile.

The strong relationship between AOL and the CHC profile was corroborated by a multiple factor analysis where the 71 hydrocarbon proportions (of total composition), AOL, and JH titer were plotted against the first two principal components on a correlation circle graph (Fig. 6b). JH titer, on the other hand, showed a strong positive relationship with the second principal component (Fig. 6b), indicating a weaker correlation with the hydrocarbons due to their stronger correlations with the first dimension. When JH titer and each hydrocarbon proportion were compared independently, only 14 of 71 comparisons showed a significant correlation, most of which (12/14) presented a much weaker level of significance than that between AOL and the hydrocarbon proportion (Fig. 3b).

Fig. 4 Principal coordinate analysis (PCoA) of CHC profiles of *P. smithii* (**a, b**) and *P. dominula* (**c, d**) foundresses. Axes are represented by principal coordinates. The centroid is represented by a *black circle* for each group, the *dashed lines* encompass all individuals of the group, and the *lines* connect the sample to the group centroid. **a** *P. smithii* social foundresses (lone foundresses excluded) with large oocytes (*triangle*) do not separate from foundresses with small oocytes (*circle*). **b** *P. smithii* foundresses grouped according to status do not cluster separately. Foundress types: lone (*circle*), alpha (*plus sign*), and subordinate (*triangle*). **c** *P. dominula* foundresses with large oocytes (*triangle*) separate from foundresses with small oocytes (*circle*). The *black arrow* indicates the only alpha foundress with small oocytes. **d** Alpha foundresses (*circle*) of *P. dominula* do not have a distinct profile from that of subordinate ones (*triangle*)



Discussion

In this study, we asked whether and how two geographically coexisting paper wasps, one indigenous and one invasive, with similarity in their social biology (both being primitively eusocial with the option to form multiple foundress associations) differ in their endocrine and chemical signatures, and also how these aspects relate to dominance and reproductive status. The main results were that in late pre-emergent colonies of *P. smithii* and *P. dominula*, JH titers showed little if any relationship to oocyte length. Instead, JH titers differed according to social status. Yet whereas JH was positively associated with dominance in *P. dominula*, in *P. smithii*, JH was, if anything, negatively associated with dominance. Ecdysteroid titers were extremely low in all foundress groups, indicating that they probably do not have a physiological function in the late pre-emergent colony stage. Finally, the cuticular hydrocarbon (CHC) profile of *P. smithii* showed no association with social status or fertility potential, and none of the hydrocarbon peaks showed a positive association with oocyte length. This is in stark contrast to the intracolony differences expressed in CHC profiles of other paper wasps (Monnin 2006; Claudia et al. 2010;

Kelstrup et al. 2014a, b; Mitra and Gadagkar 2014), including *P. dominula*, which we show here to have an undeniable link to oocyte length, i.e., fertility potential.

It is doubtful that *P. smithii* uses facial markings to advertise quality or signal identity given the lack of conspicuous, variable patterns. This result fits with the hypothesis (Sheehan and Tibbetts 2011) that such signals are more likely to evolve in species which form large foundress groups (e.g., *P. dominula*, *Polistes exclamans*, *Polistes fuscatus*, and *Polistes satan*) as opposed to species where foundresses typically (*Polistes metricus*) or frequently (*Stellenbosch P. smithii*) nest alone. In *P. dominula*, body size and the importance of facial brokenness in social interactions vary geographically, with warmer climate populations (e.g., Italy) tending to produce smaller foundresses with less variable facial patterns than larger foundresses adapted to survive colder winters (e.g., northern USA) (Tibbetts et al. 2011c). Based on the mildness of the Stellenbosch winter, where the average daily low temperature was higher than for the Italian population studied (Tibbetts et al. 2011c), we expected the Stellenbosch wasps to be smaller and to show minimal variation in facial markings. Yet in both attributes, they were intermediate to the Italian and northern USA

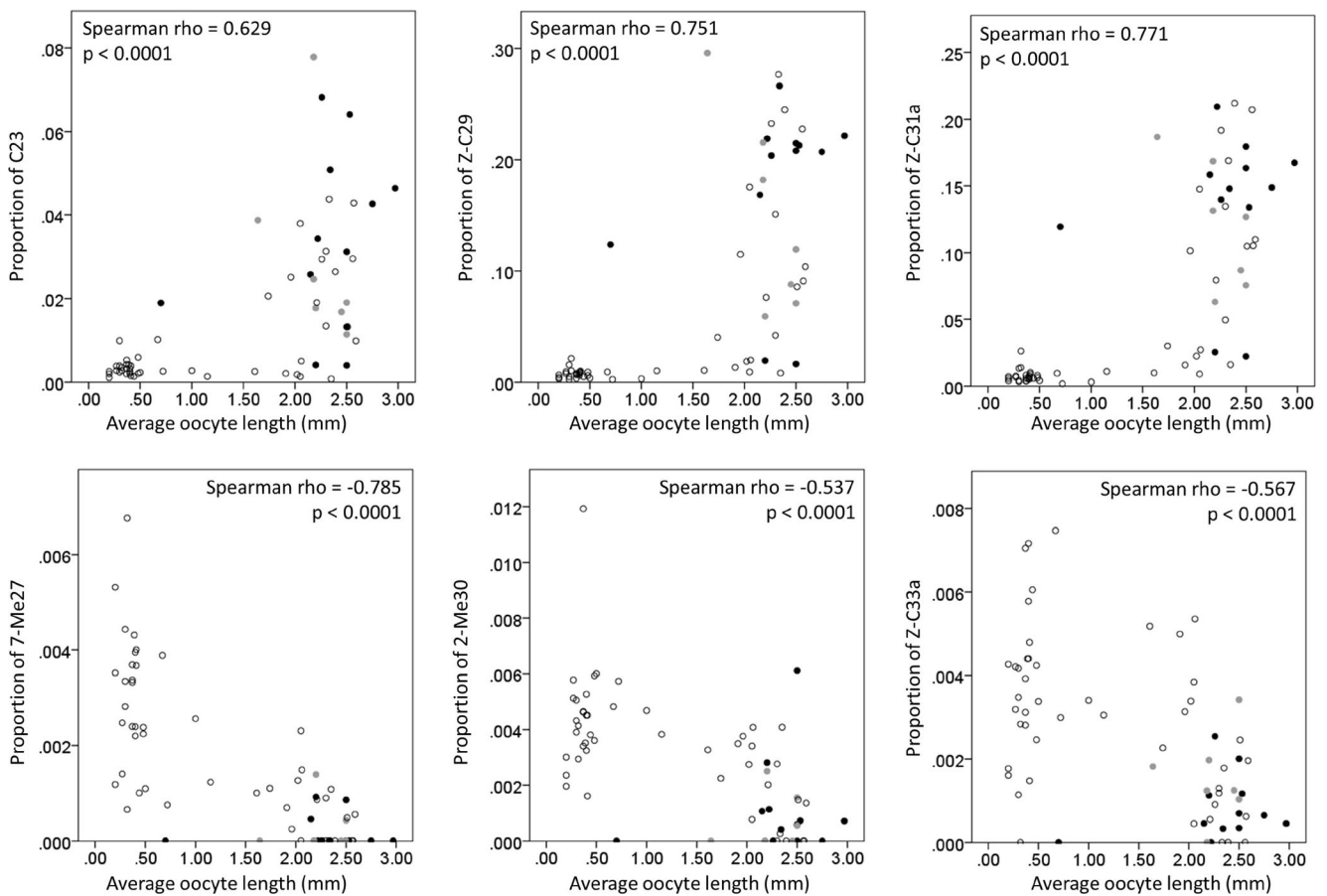


Fig. 5 Relationship between average oocyte length (AOL) and the proportional representation of six hydrocarbons of *P. dominula* foundresses. Each hydrocarbon contributed >2.5 % to the total variance and showed a highly significant association with AOL. Alphas ($n=12$),

betas with developed ovaries ($n=7$) (i.e., immediate rivals to the alphas), and all other foundresses ($n=57$) are represented by *closed black circles*, *closed gray circles*, and *open circles*, respectively

populations. Furthermore, this suggests that some of the intergeographic variation may be due to genetic differences and possibly indicates that the South Africa propagule may not have originated from a Mediterranean source population. While this needs to be resolved by population genetic marker analysis, it also remains to be seen if the South African population, which is a very recent invader, will eventually evolve smaller bodies and show a reduction in facial markings. The use of facial markings as a signal of agonistic ability is particularly important in the springtime when emerging foundresses first encounter one another. Later in the colony cycle, after linear hierarchies are established and stable, the relationship between facial pattern and dominance is better explained by other factors, such as JH titer (Tibbetts et al. 2011a). Nonetheless, the result that facial brokenness failed to show any overt association with dominance among foundresses makes us wonder whether they are truly used as signals of agonistic abilities in the earliest colony stages in the South African population.

Coexisting *Polistes* species differ in their endocrine signatures for social and ovarian status

How can the differences in the endocrine profiles of *P. smithii* and *P. dominula* be explained? First, the fact that dominant and subordinate foundresses of *P. smithii* did not differ in the level of circulating JH does not rule out the possibility that JH is fueling separate functions based on nutritional state (West-Eberhard 1996). For example, subordinate females, which lose out in food exchange interactions, are likely to expend more time and energy working due to aggression received from dominant nestmates (O'Donnell 1998). Given the apparent stimulatory role of JH in worker tasks in *P. canadensis* (Giray et al. 2005), *P. dominula* (Shorter and Tibbetts 2009), and other eusocial Hymenoptera (O'Donnell and Jeanne 1993; Robinson and Vargo 1997; Penick et al. 2011; Dolezal and Brent 2012), the JH titers of subordinate foundresses of *P. smithii* could thus reflect a nutrition-dependent function for JH. More detailed and longer-term behavioral observations of subordinate foundresses would help identify what function, if

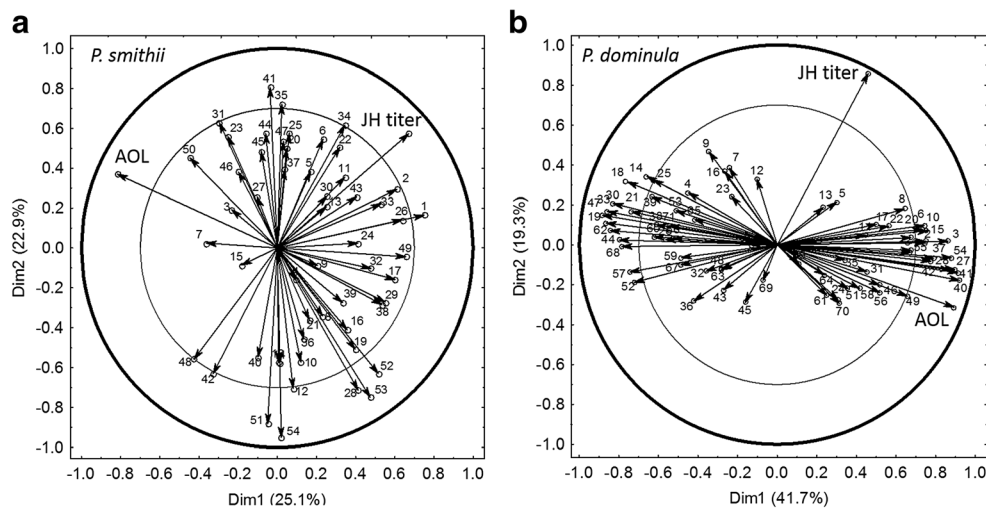


Fig. 6 Correlation circle graphs based on multiple factor analysis for *P. smithii* and *P. dominula*. Hydrocarbons are indicated by their number (see Tables S1 and S2) along with JH titer and average oocyte length (AOL). Lines stretching to outer circle indicate strong correlations (Pearson's $r > 0.7$; see axes) between the particular variable and the first two principal components (called dimensions (Dim) 1 and 2). **a** In *P. smithii*, JH titer and AOL show a strong positive and negative correlation with Dim1, respectively, and JH titer showed a stronger

correlation with Dim2. **b** In *P. dominula*, AOL and many hydrocarbon proportions show a strong positive correlation (right side of outer circle) with Dim1, while other hydrocarbon proportions show an equally strong negative correlation (left side of outer circle). Although JH titer is strongly correlated with Dim2, it is not accompanied by other variables, indicating that JH titers do not show a strong relationship with AOL and any hydrocarbon proportions

any, JH has in reproductively suppressed females. Even so, if JH were also involved in dominance and/or reproductive growth in *P. smithii*, one would expect JH titers to be elevated in alpha foundresses compared to solitary ones and, furthermore, to be higher still in alpha foundresses sharing a nest with reproductive subordinates (i.e., rivals). Yet no such pattern was evident, suggesting that JH may not be important for dominance or reproduction in *P. smithii*, at least in the late pre-emergent phase.

Clearly, the findings for *P. smithii* stand in contrast with those for *P. dominula*, in which JH titers (or indication of such) are significantly higher in alpha foundresses than their subordinates (Röseler et al. 1984, 1980; Tibbetts et al. 2011a; present study). Moreover, we show that JH titers were higher in alpha females heading larger colonies (i.e., with a greater number of potential rivals). The result that beta and lower-ranking foundresses with high fertility potential had JH titers similar to those of foundresses with low fertility potential suggests that in this species, JH is indeed more closely associated with dominance than with a static measure of ovary condition, such as average oocyte length. This lack of correlation can be explained by the presence of a class of subordinate females with large oocytes. As the subordinate females appear to fall into two distinct groups, with either small or large oocytes, viz. low and high fertility potential, and there are hardly any intermediates, it seems as if ovarian activity in the high fertility potential subordinates has “escaped” the queen dominance signal or, in other terms, that oocyte growth in this group is uncoupled from JH. On first view, such uncoupling also appears to be the case in *P. smithii*, as indicated by a trend toward

a negative correlation between JH titer and AOL, although the negative relationship was weighted by the inclusion of co-occurring lone foundresses (with high fertility) which had low JH titers. Indeed, when grouping alpha foundresses together with their subordinates, they had higher JH titers than solitary foundresses (one-way ANOVA: $F_{1,43}=7.21, p=0.01$). Interestingly, a difference between social and solitary foundresses was also described by Sledge et al. (2004) when analyzing CA volume in pre-emergence foundresses of *P. dominula*.

The virtual absence of ecdysteroids in the hemolymph in both species leads us to conclude that they are not important for foundress physiology and behavior in the late pre-emergent stage. This stands in contrast to the pre- and early-nesting phases, when ecdysteroid titers are reported to show a precipitous rise and to correlate with oocyte length (Strambi et al. 1977; Röseler et al. 1984, 1985). Manipulation studies have shown that although ecdysteroids can augment fighting ability at this stage, ovariectomized females (confirmed to lack ecdysteroids in the hemolymph) can still become the most dominant female on the colony (Röseler and Röseler 1989). Nonetheless, they fail to eat the eggs of their subordinates, a caste-defining behavior performed by normal alpha females, which left open the possibility that ecdysteroids influence some aspects of reproductive dominance (Röseler and Röseler 1989; Monnin 2006). Our results show that this is unlikely to be the case, for although we confirmed here that the ovaries of egg-bearing females of *P. dominula* are replete with ecdysteroids, their release into the hemolymph appears to be restricted to a phase where egg-eating by dominants is still

common. Similar results were found for two primitively eusocial (caste-flexible) swarm-founding paper wasps, *S. surinama* and *P. micans* (Kelstrup et al. 2014a, b). Thus, even more puzzling than JH endocrinology, understanding the role played by hemolymph ecdysteroids in paper wasp biology clearly will require more endocrine studies of *Polistes* species, as well as members of more ancient wasp clades (Pickett and Carpenter 2010).

Cuticular hydrocarbons bear a strong reproductive signature in *P. dominula*, but not in *P. smithii*

Consistent with previous reports for *P. dominula*, there were clear differences in the CHC profile between alpha foundresses and subordinates with low fertility potential, with two alkenes in particular, Z-C29 and Z-C31, being much higher in the alphas. This is consistent with the results from Dapporto et al. (2004), in which alpha foundresses bore a higher proportion of alkenes with 29 and 31 carbon-chain lengths in two of three sites. There is, however, controversy as to whether intracolony CHC profile differences relate to dominance (Dapporto et al. 2004, 2007a), fertility (Izzo et al. 2010), or both. Dapporto et al. (2010) argued that the nature of the signal may depend on the phase of the colony cycle, with possible fertility-associated cues being present on the cuticle early (when foundresses are first gathering around potential nesting sites) but then signaling dominance rank in the later phases. Nonetheless, our results clearly support the hypothesis that the CHCs are more important in conveying information about fertility in the late pre-emergent phase, at least in South Africa. Whereas splitting females into two groups based on high and low fertility potential showed obvious differences in the CHC profile, there was no difference when females were categorized according to social status, especially so when the alpha and beta foundresses had comparable ovaries (e.g., both types of foundresses had elevated proportions of Z-C29 and Z-C31 on their cuticle). Yet among lower-ranking foundresses, it was also evident that some females with high fertility potential were not distinguishable from those with lower fertility potential (see group overlap in Fig. 4c). Also, an alpha foundress with small, borderline vitellogenic oocytes had a very high proportion of the above alkenes and therefore grouped more closely to fertile foundresses, indicating that the hydrocarbon profile is not a simple byproduct of fertility (as has been argued for another paper wasp; see below) and may, in some cases, convey dominance rank. It would thus be interesting to determine the CHC profiles of alphas which remain dominant after their ovaries are removed.

Untangling prospective fertility and dominance rank cues of the CHCs has been notoriously difficult in *P. dominula*, and when it has been explicitly and experimentally tested in established pre-emergent nests from Italy, dominance rank has been identified to be a much more important contributor

to the CHC profile than fertility (Dapporto et al. 2007a). Our results offer no support for the hypothesis that alpha foundresses have a unique chemical signature compared to equally fecund subordinates, and so the high JH titers of alphas are unlikely to be of primary importance in directly affecting the CHC profile. Notwithstanding, it should be kept in mind that the relative contributions of fertility and social status to the CHC profile of *P. dominula* may vary across populations, just as the contributions of specific hydrocarbons do. For instance, alkenes of C29 and C31 have been shown to be absent in some populations (Dapporto et al. 2004).

One of the more surprising results from the present work was the lack of a prospective fertility or dominance hydrocarbon signature on the cuticle of *P. smithii* foundresses, although the sample size was small. In most eusocial Hymenoptera which are caste-flexible, fertility and/or dominance is almost always linked to differences in the CHC profile (Monnin 2006; Liebig 2010; Richard and Hunt 2013), as was shown here again for *P. dominula*. This also holds true for several species of hover wasps (Stenogastrinae), the most basal subfamily of eusocial vespids (Turillazzi et al. 2004), and other paper wasp species studied to date (Monnin 2006; Tannure-Nascimento et al. 2008; Liebig 2010; Mitra and Gadagkar 2012; Richard and Hunt 2013). In other species, the fertility or dominance-related differences in the CHC composition are much more nuanced (Espelie et al. 1994; Toth et al. 2014), and it may be worthwhile to reevaluate these data with PerMANOVA methods.

The conclusion that the CHCs of *P. smithii* do not contain information relevant to fertility or dominance does not mean that distinctive chemical signals are not being produced. For example, dominant foundresses may dispense a distinct composition of chemicals from specialized glands which are rubbed onto the nest to indicate the presence of a fertile dominant (Dapporto et al. 2007b; Mitra and Gadagkar 2012). In species where both glandular and cuticular hydrocarbons have been compared, however, there is good correspondence between these profiles (Dani et al. 1996, 2003; Dani 2006; Mitra and Gadagkar 2011, 2014). Mitra and Gadagkar (2014) also showed that the hemolymph of *Ropalidia marginata* contains the same hydrocarbons in similar abundances as the cuticle and Dufour's gland, suggesting a common source of synthesis (e.g., oenocytes of the fat body) and mode of conveyance. Yet in *P. metricus*, where CHC profiles show only a slight association with fertility or dominance, obvious status-associated differences were detected in several organs (Dufour's, sternal, and mandibular glands) containing an unknown medley of compounds (Toth et al. 2014). Besides chemical signals, and in addition to behavioral ones, the prolonged presence of empty brood cells on the comb may itself reveal low fertility of the dominant foundress, compelling subordinates to produce eggs despite her presence (Liebig et al. 2005; Dapporto et al. 2007b).

Conclusions

While overarching conclusions about the evolution of JH, ecdysteroid, and CHC functions (aside from nestmate recognition) within the genus *Polistes* may still be beyond reach, the variation already evident from comparing patterns in a handful of species suggests that their roles are not well conserved or constrained. Our results show that even closely related paper wasp foundresses sharing the same environment show divergent endocrine and CHC profiles. This may be due to a number of reasons. First, the distinct biographic histories of the species should be considered, since *P. smithii* and *P. dominula* evolved a diapause-based colony cycle in different temperate zones. Fortunately, we should be able to assess whether changes in endocrine physiology preceded or followed the evolution of the diapause-foundress phenotype in *P. smithii* by studying tropical populations which occur throughout sub-Saharan Africa (Carpenter 1996). Second, species- and population-specific differences in social characteristics, such as the tendency for foundresses to nest solitarily, in small groups or large ones, and whether or not fecund subordinates may be present, are likely to have an effect on the endocrinology of the individual. Even within *P. dominula*, there are notable geographic-specific differences in the social structure of foundress nests, with some invasive colonies containing many egg-laying females (Liebert et al. 2008; this study), in stark contrast to native populations in Europe where only one or few foundresses are typically present (Pardi 1942). Future studies on paper wasp endocrinology, both within and between species, should aim to control for the number of individuals on the nest in order to separate out whether differences in hormone levels, chemical profiles, and facial patterns relate more to social factors, environment (e.g., geography and climate), or population (i.e., genetics).

When putting the results for the two primitively eusocial South African *Polistes* species together with those obtained for three Neotropical species, the swarm founding, caste-flexible *P. micans* and *S. surinama* (Kelstrup et al. 2014a, b) and the casteless nest-sharing *Zethus miniatus* (manuscript in preparation), it became apparent that JH, social status, ovarian activity, and CHC patterns are related but not necessarily always with the same directionalities. Especially for JH, the emerging picture is that of functional variability, meaning that a phylogenetically ancient and pleiotropic hormone regulating insect metamorphosis and reproduction has apparently been put to use in various contexts of social lifestyles, not only across orders, families, or genera but also within a single species. *P. dominula* appears to be a particularly illustrative case, whereby face marks expressing dominance potential and JH levels are correlated with ovary size in the earliest phase of post-diapause colony foundation. Subsequently, in the late pre-emergence phase, when a dominance hierarchy is already established, JH levels and dominance continue to be related,

but ovarian activity appears to become disconnected from JH, at least in the subordinate females. The question then becomes, and there is insufficient data so far, what happens as a multiple-foundress colony gradually transits to a truly eusocial mother/daughter condition? Does JH then also become decoupled from the queen's dominance position to gain a function in age-related division of labor among the workers? Possibly, but most probably not in all species, since what we may be seeing here is the evolutionary tinkering with connectivities in hormonal signaling, the MEKRE93 pathway (Belles and Santos 2014), made possible exactly because of the pleiotropism of JH, especially so in social insects.

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