A novel property of spider silk: chemical defence against ants

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Spider webs are made of silk, the properties of which ensure remarkable efficiency at capturing prey. However, remaining on, or near, the web exposes the resident spiders to many potential predators, such as ants. Surprisingly, ants are rarely reported foraging on the webs of orb-weaving spiders, despite the formidable capacity of ants to subdue prey and repel enemies, the diversity and abundance of orb-web spiders, and the nutritional value of the web and resident spider. We explain this paradox by reporting a novel property of the silk produced by the orb-web spider Nephila antipodiana (Walckenaer). These spiders deposit on the silk a pyrrolidine alkaloid (2-pyrrolidinone) that provides protection from ant invasion. Furthermore, the ontogenetic change in the production of 2-pyrrolidinone suggests that this compound represents an adaptive response to the threat of natural enemies, rather than a simple by-product of silk synthesis: while 2-pyrrolidinone occurs on the silk threads produced by adult and large juvenile spiders, it is absent on threads produced by small juvenile spiders, whose threads are sufficiently thin to be inaccessible to ants.

Keywords: facultative chemical defence; orb web; risk of predation; spider silk; ant deterrent; 2-pyrrolidinone

1. INTRODUCTION
Chemical weaponry, including repellent or deterrent odours that reduce the probability that the defending individual is killed or loses its resource, is a common defence mechanism among the myriad adaptations that have evolved in response to selection by natural enemies [1,2]. Among the most widespread forms of defensive chemicals are pyrrolidine alkaloids, a class of defense compounds that many arthropods acquire from their food or biosynthesize, which are toxic or unpalatable to a vast array of potential predators [1–3]. Producing these chemical defenses may be costly, perhaps reflected by the close link between their expression and the risk of predation at the population level [4] or following enemy attack at the individual level [5]. Interestingly, individual adjustments of chemical defence mechanisms according to the risk of predation are not widely documented—in contrast with, for example, the vast literature on behavioural adjustments to predation risk [6].

The silk produced by orb-web spiders (Araneoidea) has extraordinary properties (including high strength, elasticity and adhesion) that allow the web to be a highly efficient means of arresting prey [7,8]. Many orb-web spiders store the remains of prey in their webs [9], and these items, together with other prey entangled in the web, the protein-rich composition of silk [8] and the resident spider itself, provide nutritional resources for predators and scavengers, particularly small kleptoparasitic Argyrodes spiders [10,11]. Ants can be major predators of spiders [12–14], and workers of some species invade spider webs, establishing foraging trails on the non-sticky silk strands to access large caches of food [15,16]. Nevertheless, ants are rarely reported foraging on the webs of orb-web spiders, despite their diversity, abundance and vulnerability during molting [17–20], and the superabundance and formidable predatory capabilities of ants [21].

Golden orb-web spiders Nephila antipodiana (Nephilidae) are found in forested areas, where they construct a prey-capturing orb-web within a support frame comprised of scaffold silk threads. The orb-web, which sustains damage and accumulates small prey items, is consumed and renewed by the resident spider every few days. The supporting silk frame can persist for several weeks or more, and ants intending to invade the orb-web must first traverse these silk threads. While the structural properties of the silk, particularly the diameter of the threads, may prevent large ants from traversing the silk, other defences such as chemical deterrents may be necessary for smaller ants. The pharaoh ant, Monomorium pharaonis (L.), is a widespread, abundant pest [22], and the very small workers are predators of spiders [21,23]. Pharaoh ants live in forested areas where N. antipodiana is found, including at our study site.

Here, we reveal a novel role of chemicals on the web silk of N. antipodiana in providing defence against ants, and in particular that these chemicals are deployed in response to increased risk of attack by ants. We report the presence of a pyrrolidine alkaloid, 2-pyrrolidinone, on the silk of the orb-web spider N. antipodiana and demonstrate that it deters workers of three species of myrmicine ants. Furthermore, we show that 2-pyrrolidinone is present on the silk threads produced by adult and large juveniles, but absent on threads produced by small juveniles, whose threads are
sufficiently thin to be inaccessible to ants. We conclude that the production of 2-pyrrolidinone is an adaptive response to the threat of natural enemies, rather than a simple by-product of silk synthesis.

2. MATERIAL AND METHODS
(a) Study subjects
We collected adult females, and large (body length > 4.1 mm) and small (body length < 4.1 mm) juveniles of N. antipodiana from various sites (Kent Ridge Park, National University of Singapore, Labrador Nature Park) in Singapore. They were maintained individually in plastic frames (females and large juveniles: 100 × 100 × 30 cm; small juveniles: 30 × 30 × 30 cm) in the laboratory under controlled environmental conditions (temperature: 25 ± 1°C; relative humidity: 80 ± 10%; photoperiod: 12 L: 12 D cycle, with lights on at 08.00 and off at 20.00). Spiders were allowed to build their webs in the frames in the absence of any prey, thereby ensuring that any compounds extracted from the silk were not contaminated by the prey that had encountered the web. No spiders were used more than once.

(b) Chemical analysis
We used gas chromatography–mass spectrometry (GC-MS) to identify the chemicals on the surface of the silk. We obtained silk from newly constructed spider webs that were uncontaminated by prey items, using sterilized glass rods (Fisher Scientific, Pittsburgh, PA). We collected silk from single webs built by adult or large juvenile females (n = 21) or small juveniles (n = 20). The webs of adult and large juvenile spiders provide plenty of silk for each sample. However, small juvenile spiders produce less silk and we employed three methods to ensure that an absence of ant-deterrent compounds on the silk produced by these spiders was not an artefact of small quantities of silk. Thus, for each method, we obtained two samples, each comprising (i) 2.5 mg silk spun by one or a few individuals of large juveniles (4.2–7 mm body length), (ii) 2.5 mg silk spun by multiple small juveniles (2.1–4 mm body length) and (iii) silk produced by 10 different small juvenile spiders ranging from 1.8 to 3.7 mm in body length. Each silk sample was placed in a 10 ml glass vial (Supelco, Bellefonte, PA) with 1 ml of methanol, and solvent extraction involved shaking the vial with the silk in the solvent for 10 min. The extract was then concentrated by blowing nitrogen gas over the surface of the extract until the total amount of extract was 10 μl. Two microlitres of each extract was inserted into a GC-MS system, using a 5R-GP syringe (SGE, Ringwood, Victoria, Australia). The mass spectra were obtained with a HP GC 6890+ series coupled with a MS 5973 detector. Zebron (ZB-1) polymethylsiloxane columns of 30 m × 250 μm × 0.25 μm capillaries (Phenomenex, Torrance, CA) with Helium carrier gas of 1.5 ml min⁻¹ were used. The injector port and detector temperature were maintained at 280 °C. The separation protocol, developed to ensure good separation of the compounds for the analysis, started with an initial temperature at 50 °C for 3 min, then increased to 280 °C at a rate of 4 °C min⁻¹ and was held at that temperature for 2 min. Subsequent comparative analysis was conducted using the NIST02 and WILEY 275L database for possible identification of any chemicals. The identified compound was compared with the reference compound (99% 2-pyrrolidinone, GC grade; Sigma-Aldrich, Bellefonte, PA). Nitrogen-gas-concentrated methanol alone was used as a negative control to establish that no similar peaks or ions of the compound were present in the spectrum.

We determined the concentration of 2-pyrrolidinone on the web silk by obtaining 2.34–9.37 mg of silk from each individual web of adult or large juvenile females (n = 7), and immersing the silk in methanol for 20 mins to extract 2-pyrrolidinone. We diluted the synthetic standard sequentially in methanol to concentrations of 0.1, 1, 10, 50 and 100 mg ml⁻¹, and 1 μl of each prepared solution was injected into the GC-MS system to obtain the calibration regression equation. The calibration regression equation is y = 517.666x + 1150.505 (r² = 0.99751).

(c) Behavioural bioassays
Behavioural bioassays were conducted to test whether the diameter of the silk or the presence of 2-pyrrolidinone deters ant workers from the silk threads produced by N. antipodiana. We initially used a natural population of pharaoh ants, M. pharaonis (body length: 1.5–2 mm), located in the laboratory. To confirm that 2-pyrrolidinone acts as a general deterrent, we tested two other species of ants, Pheidole angulicollis Eguchi (body length: 1.82 ± 0.03 mm, n = 25) and an unidentified species of Monomorium (body length: 1.42 ± 0.03 mm, n = 25), in field locations where N. antipodiana occurs.

The bioassay apparatus consisted of a platform with a baiting station and three silk bridges: the sides of the baiting station were coated with a layer of Fluon (Northern Products Inc., Woonsocket, RI), which ensured that the silk bridges provided the only access to the bait (figure 1a). Each bridge (3 cm) comprised silk from the scaffold thread of

Figure 1. The bioassay chamber and ants crossing the silk bridge. (a) The bioassay chamber. Workers had access to and from the food source via different silk bridges that were constructed from the scaffold threads of webs of Nephila antipodiana. (b) A photo showing two Pheidole angulicollis workers crossing the bridge (deterrent removed; top) constructed with the silk threads produced by adult Nephila antipodiana and one ant retreating upon contacting the bridge constructed with natural silk (control; bottom) produced by adult N. antipodiana.
the webs of *N. antipodiana* that had been spun in the laboratory. Scaffold thread collected from each web was divided into five 3 cm samples: three samples were used as silk bridges for behavioural bioassays and the other two samples for measuring the diameter of the threads (see later text). The three silk bridges used in each trial were randomly allocated to one of three treatments: deterrent removed, deterrent added and control (i.e. natural silk thread). The surface chemicals on the silk threads for the deterrent removed and deterrent added treatments were first removed by immersing the silk in 70 per cent alcohol for 2 h, with the solution changed every half an hour, and subsequently baking the silk threads in the oven (70°C) for 48 h. In this way, there is an internal control, and the natural silk control provides a comparison. Starch was used to adhere the three silk bridges to the baiting station, and the complete apparatus was positioned adjacent to an ant colony. Freshly killed *Musca domestica* and *Drosophila melanogaster* were placed at the baiting station to attract ants, who could access the bait via the silk bridges. All the silk bridges except the control were used by ants before either the 2-pyrrolidinone dilution or detergent removed and deterrent added treatments were first removed by immersing the silk in 70 per cent alcohol for 2 h, with the solution changed every half an hour, and subsequently baking the silk threads in the oven (70°C) for 48 h. In this way, there is an internal control, and the natural silk control provides a comparison. Starch was used to adhere the three silk bridges to the baiting station, and the complete apparatus was positioned adjacent to an ant colony. Freshly killed *Musca domestica* and *Drosophila melanogaster* were placed at the baiting station to attract ants, who could access the bait via the silk bridges. All the silk bridges except the control were used by ants before either the 2-pyrrolidinone dilution or distilled water was applied. After 2 h, when between 150 and 200 ants had accumulated around the prey, 1.5 µl of a 2-pyrrolidinone dilution (0.05 mg dissolved in distilled water) was applied to the deterrent added silk bridge, while 1.5 µl of distilled water was applied to each of the silk bridges of the deterrent removed and control treatments. We recorded the behaviour of ants over the next 2 h, using a video recorder (Sony HDR-SR8), noting the number of ants that crossed each bridge. The location of the silk bridge treatments was randomized across trials, and the silk bridges and all of the ants were destroyed at the end of each trial to prevent recognition and learning of chemical cues. The arena was cleaned with 70 per cent ethanol and allowed to dry for about 30 min between trials. All trials were carried out between 07.00 and 18.00.

The scaffold silk used to support the orb-web may be single or multi-stranded; so we conducted two experiments using workers of *M. pharaonis*. In the first experiment, each silk bridge comprised several threads of silk (15 trials), and in the second experiment, each bridge comprised a single silk thread (5 trials).

We examined whether the deterrent effect of 2-pyrrolidinone persists over a lengthy time period by repeating the experiments using silk of different ages. The scaffold threads were collected and prepared as described earlier, and stored under ambient laboratory conditions for either 15 or 30 days. We then conducted the bioassays, as described earlier, using workers of *M. pharaonis*.

**d) Spider body size and web silk diameter**

We examined the relationship between the size of the spider and the diameter of her silk threads. We measured the diameters of single-stranded and multi-stranded web silk threads under a scanning electron microscope (JSM-6510LV, JEOL, Tokyo, Japan). For each web, we collected two 3 cm samples of scaffold silk threads directly onto a piece of cardboard: one for measuring the diameter of a single-stranded thread and the other for a multi-stranded thread. We took five haphazardly selected measurements for each silk sample. In addition, we also measured body length for each spider that built the web from which the silk samples were obtained.

**3. RESULTS**

The compound 2-pyrrolidinone (figure 2) was detected on the silk of all webs constructed by adults and large juveniles (body length > 4.1 mm, *n* = 21) of *N. antipodiana*, but not on the silk produced by small juveniles (body length < 4 mm, *n* = 20). The quantity of 2-pyrrolidinone on the silk of adult and large *N. antipodiana* (*n* = 7) was determined at a mean (± s.e.m) concentration of 6.15 (± 1.19) µg mg⁻¹.

The absence of 2-pyrrolidinone on silk produced by small juvenile *N. antipodiana* was not an artefact of
small samples of silk: 2-pyrrolidinone was present in both of two samples of 2.5 mg silk spun by large juveniles, but absent in both of two samples of 2.5 mg silk produced by small juveniles as well as both of two samples of silk produced by 10 small juveniles.

Pharaoh ant workers frequently traversed bridges of silk threads without 2-pyrrolidinone (deterrent removed), but rarely crossed silk bridges with 2-pyrrolidinone (deterrent added and control; figure 3 and table 1). This pattern emerged irrespective of whether the bridge was constructed of multiple silk threads (Fisher’s exact test: \( p < 0.01 \); different letters indicate significance differences). Workers coming into contact with silk that had been treated with 2-pyrrolidinone rapidly retreated, but we did not observe any instant toxic effects on the workers that contacted 2-pyrrolidinone (body length: 1.6–2.1 mm) and Monomorium sp. (body length: 1.3–1.6 mm). The mechanical properties of silk also affected whether pharaoh ants traversed the silk threads. Bioassays with bridges made of scaffold silk threads produced by spiders of different sizes (ANOVA: \( F_{2,15} = 77.753, p < 0.0001 \); figure 4a) and from which the deterrent had been removed revealed that pharaoh ant workers can cross the bridges constructed with silk threads produced by adults and large juveniles, but not the bridges constructed with silk threads produced by small juveniles (Fisher’s exact test: \( p < 0.0001; n = 5; \) figure 4b). This difference is probably due to the diameter of the silk bridge, which depends upon the size of the spider: the silk thread of adult females and larger juveniles is four to seven times thicker than that of small juveniles, irrespective of whether the bridge is constructed of multiple silk threads (ANOVA: \( F_{2,15} = 14.988, p < 0.01; \) figure 4c) or single silk thread (ANOVA: \( F_{2,15} = 54.67, p < 0.01; \) figure 4d).

Table 1. The influence of the presence and absence of 2-pyrrolidinone on the number of Monomorium pharaonis workers that crossed different kinds of silk bridges.

<table>
<thead>
<tr>
<th>number of ants</th>
<th>deterrent added</th>
<th>deterrent removed (min(^{-1}))</th>
<th>control (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>several strands of silk from adult spider</td>
<td>0</td>
<td>14.8 ± 1.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>single strand of silk from adult spider</td>
<td>0</td>
<td>16.2 ± 1.4</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. The effects of silk age on the number of trials in which at least one M. pharaonis worker crossed the bridges constructed with multiple silk threads produced by adult Nephila antipodiana females and subject to different treatments (see text).

<table>
<thead>
<tr>
<th>age (days) of silk</th>
<th>deterrent added</th>
<th>deterrent removed</th>
<th>control</th>
<th>Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ((n = 5))</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>( p = 0.009 )</td>
</tr>
<tr>
<td>15 ((n = 5))</td>
<td>0</td>
<td>5</td>
<td>1</td>
<td>( p = 0.006 )</td>
</tr>
<tr>
<td>30 ((n = 5))</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>( p &lt; 0.0001 )</td>
</tr>
</tbody>
</table>

4. DISCUSSION

This is the first report of the production of chemical components on spider web silks that act as a deterrent against...
natural enemies, although similar properties have been widely suspected [24], but with mixed empirical support [25,26]. The ontogenetic or size-dependent addition of 2-pyrrolidinone is significant because it suggests that the compound represents an active defense mechanism, rather than a general by-product of silk production: while 2-pyrrolidinone is present on the webs of adult and large juvenile spiders that are potentially vulnerable to ant invasion, it is absent on the silk produced by small spiders—presumably because their silk threads cannot support the movement of ants and thus do not require a chemical deterrent. While it is possible that other features of the silk, which might be altered by our experimental treatments (i.e. the solvent extraction and 48 h baking at 70°C), may also act as ant deterrents, our data indicate that 2-pyrrolidinone is the most important.

The expression of 2-pyrrolidinone on silk according to the risk of natural enemies may reflect the costs of synthesizing this chemical deterrent, together with the metabolic or energy constraints faced by small spiders. Such phenotypic plasticity in the production of chemical defences in response to the risk of natural enemies is common in plants [27], but not widely reported in terrestrial invertebrates [5]. According to coevolutionary models of predator–prey
interactions, we predict that 2-pyrrolidinone will be absent on silk produced by orb-weaving spider species that are typically less than 4 mm long or for which the silk threads have a mean diameter of less than 3.06 μm. Conversely, 2-pyrrolidinone may not necessarily act as a deterrent to large species of ants, whose size might prevent them from being able to traverse the silk produced by even large species of orb-weavers.

It is likely that 2-pyrrolidinone serves principally as a contact deterrent than as a volatile repellent: the ants in the behavioural assays remained in the vicinity of the silk, avoiding it only when they came into direct contact. A contact deterrent is resource-efficient because, as our experiments reveal, it lasts for an extended time, which may be significant for those species in which the silk threads used to anchor the web to the vegetation are not rebuilt everyday. Chemical deterrents may also be more effective against ants than other defense mechanisms against solitary enemies; for example, orb-web spiders may physically chase kleptoparasitic spider intruders (e.g. Argyrodes) off the web [11], but this may be ineffective against a large number of recruiting ants. However, physical defence may be more efficient in species in which numerous spiders are present on a single web, and this may explain why ants are apparently not deterred from venturing onto the webs of social spiders [16].

Large individuals of N. antipodiana may employ 2-pyrrolidinone as a prophylactic defense against ants, rather than as a means of disabling potential prey via its toxic properties. Ants in our experiments that physically contacted the silk did not obviously alter their activities compared with ants that did not contact the silk, although the toxicity of 2-pyrrolidinone to ants is not known. In addition, 2-pyrrolidinone may act as a generalized deterrent and thus explain why ants rarely occur on the webs of orb-web spiders. Workers of three myrmecine ants, including an introduced species that has a global distribution, do not venture onto the silk produced by large individuals of N. antipodiana. 2-pyrrolidinone has broader biological significance in ants: it is in the extracts from the mandibular glands of the myrmicine ant Crematogaster sjostedti Mayr [28], and alarm pheromones of many species of ants are secreted from these glands [21]. Further, pyrrolidine alkaloids occur in the extracts of poison glands of several species of ants [29] and of abdominal glands of gypsy moth (Lymantria dispar L.) caterpillars, which use pyrrolidine alkaloids to deter ant predation [30].

2-pyrrolidinone is biosynthesized from gamma-amino-butyric acid (GABA) in the gypsy moth caterpillars [30]. Coincidentally, this acid occurs on the web of the congeneric N. clavipes [31] and three other species of orb-weaving spider: Araneus diadematus [32], Argiope trifasciata and Argiope aurantia [33]. While GABA and its derivatives are the principal component in the water extract of the web, their function was not tested [31]. Indeed, Schildknecht et al. [34] reported the presence of 2-pyrrolidinone on the silk web of A. diadematus (as Aranea diadema) and they speculated that it was a hygroscopic substance. It is not clear why 2-pyrrolidinone was detected in some species but not in others, but may reflect different methodologies (e.g. flame photometer used by Tillinghast & Christenson [31] and thin layer chromatography by Anderson & Tillinghast [33]) or different solvents. Nevertheless, these data suggest that 2-pyrrolidinone is used by a broad diversity of orb-weaving spiders, and raises the intriguing question of how ant-deterrents have evolved in this group.

Predatory wasps (e.g. mud-dauber wasps) are thought to be the primary selective force shaping the evolution of web architecture in aerial web-building spiders [35]. Our study reveals that predation by ants may similarly act as a novel selective force shaping silk properties. More generally, the potentially broader role of this chemical highlights how chemical defences can be phenotypically plastic, thus providing a new perspective to the role of chemical defences in predator–prey and animal–environment interactions.

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