Lepidopteran defence droplets - a composite physical and chemical weapon against potential predators

Pentzold, Stefan; Zagrobelny, Mika; Khakimov, Bekzod; Engelsen, Søren Balling; Clausen, Henrik; Petersen, Bent L; Borch, Jonas; Møller, Birger Lindberg; Bak, Søren

Published in:
Scientific Reports

DOI:
10.1038/srep22407

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Pentzold, S., Zagrobelny, M., Khakimov, B., Engelsen, S. B., Clausen, H., Petersen, B. L., ... Bak, S. (2016). Lepidopteran defence droplets - a composite physical and chemical weapon against potential predators. Scientific Reports, 6, [22407]. https://doi.org/10.1038/srep22407
Lepidopteran defence droplets - a composite physical and chemical weapon against potential predators

Stefan Pentzold 1,†, Mika Zagrobelny 1, Bekzod Khakimov 2, Søren Balling Engelsen 2, Henrik Clausen 3, Bent Larsen Petersen 4, Jonas Borch 5, Birger Lindberg Møller 1,6 & Søren Bak 1

Insects often release noxious substances for their defence. Larvae of Zygaena filipendulae (Lepidoptera) secrete viscous and cyanogenic glucoside-containing droplets, whose effectiveness was associated with their physical and chemical properties. The droplets glued mandibles and legs of potential predators together and immobilised them. Droplets were characterised by a matrix of an aqueous solution of glycine-rich peptides (H-WG 11-NH 2) with significant amounts of proteins and glucose. Among the proteins, defensive proteins such as protease inhibitors, proteases and oxidases were abundant. The neurotoxin β-cyanoalanine was also found in the droplets. Despite the presence of cyanogenic glucosides, which release toxic hydrogen cyanide after hydrolysis by a specific β-glucosidase, the only β-glucosidase identified in the droplets (ZfBGD1) was inactive against cyanogenic glucosides. Accordingly, droplets did not release hydrogen cyanide, unless they were mixed with specific β-glucosidases present in the Zygaena haemolymph. Droplets secreted onto the cuticle hardened and formed sharp crystalline-like precipitates that may act as mandible abrasives to chewing predators. Hardening followed water evaporation and formation of antiparallel β-sheets of the peptide oligomers. Consequently, after mild irritation, Zygaena larvae deter predators by viscous and hardening droplets that contain defence proteins and β-cyanoalanine. After severe injury, droplets may mix with exuding haemolymph to release hydrogen cyanide.

Defensive secretions are widespread in animals and may be delivered to attackers by spraying, spitting, stinging, biting or smearing of the substances 1. Defensive substances may consist of diverse components, such as secondary metabolites, peptides and proteins, which may have adverse effects on the physiological, locomotory, digestive or nervous system of predators 2–5 and even pathogens 6. Numerous insect species release or secrete such repellent, antinutritive or toxic compounds when attacked 7–9 often via exocrine glands 10. Some well-known examples of defensive secretions are the pygidial glands of bombardier beetles 11, the glandular secretions of juvenile Chrysomelina leaf beetles 12, the frontal glands of termite soldiers 13 or urticating hairs of many lepidopteran larvae 14. Thus, storage of toxins in special tissues and the immediate secretion of the toxin after physical irritation allow such insects to respond actively towards predator attacks.

A striking example of secretion-based defence in Lepidopterans has been reported in many species of the superfamily Zygaenoidea (e.g. burnets, foresters, slug moths) 15. These larvae possess segmentally arranged cavities as part of their extraordinary thick cuticle 15,16 (Fig. 1). Such cuticular cavities harbour highly viscous droplets known to contain cyanogenic glucosides (CNglcs) 16,17. The defence droplets are released in response to mild physical irritation and can be reabsorbed when the irritation stops 16. In aposematic Zygaena filipendulae larvae, droplet release is facilitated by contraction of segmental muscles around the irritated part of the body, which leads to an increased pressure of the haemolymph as well as within the cuticular cavity 16. As a consequence,
the cuticular cavity disrupts at its weakest part, i.e. the thin cuticular opening structure (Fig. 1), and a droplet is extruded\(^1\). Remarkably, neither specialised muscles nor specific cell types with morphological adaptations are involved in the secretion process, which renders such a defence system quite unique in comparison to other insect defence systems\(^1\); for example, Chryso melina leaf beetle larvae possess specialised muscles connected to the secretory gland to control release of defensive secretion\(^1\), and the so-called easy bleeding by sawfly larvae relies on an integument with exceptional low mechanical resistance that ruptures under mechanical stress leading to haemolymph exudation\(^1\).

In Z. filipendulae larvae, defence droplets constitute the main site for accumulation of high concentrations of the two CNglcs linamarin and lotaustralin (~25 μg per μl)\(^17\), which are sequestered from the host plant\(^20\) and/or biosynthesised\(^21\). These CNglc-containing droplets may serve at least two functions in defence against predators: as a deterrent due to their bitter taste and as an active defence due to release of toxic hydrogen cyanide (HCN) by hydrolysing the CNglcs with specific β-glucosidases after tissue damage\(^22,23\). HCN is an acute respiratory toxin to almost all eukaryotic organisms\(^24\); however, its odour or taste alone does not repel all insect enemies, e.g. not predatory ants\(^25\). Thus, the absence of a dual toxic/repellent role of HCN may have spurred evolution of additional physical and chemical defences in CNglc-containing secretions. This is exemplified by defence droplets in closely related Z. trifolii larvae, which possess strong repellent properties against various vertebrate and invertebrate predators\(^6,26\). This observation raises questions about the molecular and chemical basis of repellence and deterrence, and whether other substances and mechanisms apart from CNglcs in the defence droplets are required for their effectiveness.

In this study we used a multidisciplinary approach to advance our general knowledge on both physical and chemical defence mechanisms in insect secretions using Z. filipendulae larvae as the experimental system. The effectiveness of their defence droplets was tested on two potential predators and most of the constituents in the larvae as the experimental system. The effectiveness of their defence droplets was tested on two potential predators and most of the constituents in the defence droplets and their mode of action were identified and analysed. Our study shows that the mode of action of the defence droplets depends on the type of predator as well as on the degree of irritation or injury the larvae are subjected to.

### Results

#### Wetting capacity and viscosity of the defence droplets.

Defence droplets were able to wet the Z. filipendulae cuticle, in contrast to water (Fig. 2A,B). Similarly, a 0.1% solution of the surfactant SDS (sodium dodecyl sulfate) was able to wet the Z. filipendulae larval cuticle (Fig. 2C). The effect of defence droplets on potential predators was illustrated using the common red ant Myrmica rubra (Hymenoptera: Formicidae) and the cricket-bat orb weaver spider Mangora acalypha (Araneae: Araneidae). Defence droplets completely wet the hydrophobic cuticle of the ant head including mouthparts such as mandibles and sensory organs such as antennae (Fig. 2A'). Around five minutes after the wetting, the defence droplets became increasingly sticky and glued the ants and their appendages together and immobilized them. Similarly, the surfactant 0.1% SDS wet the ant head including the mandibles (Fig. 2C'). In contrast, water was not able to wet the head cuticle of the ant (Fig. 2B'). In experiments with M. acalypha (Fig. 2D) the defence droplets were observed to effectively glue together the legs of the spider which resulted in complete immobilisation and finally death (Fig. 2D').

#### Proteins and peptides in the defence droplets.

Since viscous secretions from the few lepidopteran species investigated are proteinaceous\(^26,27\), the protein composition of the Z. filipendulae defence droplets was determined. To tentatively characterize the proteins present in the defence droplets, LC-MS/MS (Liquid chromatography-tandem mass spectrometry) based amino acid sequencing and subsequent database searches were carried out. A total of 66 different proteins were identified (Table 1). Protease inhibitors, proteases and oxidases were the most abundant types of proteins in the defence droplets constituting 32 of the 66 identified proteins. The presence of peptides in the defence droplets was investigated using MALDI-TOF-MS (Matrix
assisted laser desorption/ionization time-of-flight tandem mass spectrometry) in the mass region 600–5000 Da. This approach revealed a single abundant peptide with a mass of 831.35 (MH\(^{+}\)) (Fig. 3A), which based on its fragmentation spectrum (Fig. 3B) was identified as the peptide H-WG 11-NH\(_2\) (i.e. tryptophan followed by an 11-omer of glycine) with a C-terminal α-amidation (Fig. 3C). The H-WG11-NH\(_2\) peptide was not detected in the larval haemolymph. A transcript corresponding to this sequence could not be found in any of the three available Z. filipendulae transcriptomes\(^{21,28}\). This indicates that the peptide in Z. filipendulae originates via post translational processing. To find a possible match for the peptide in other organisms, the sequence was searched against UniProtKB using Peptide Match\(^{29}\) and against the NCBI non-redundant protein sequences database using the BLASTP algorithm. This approach resulted in sequence hits from several different organisms with 100% coverage and 100% sequence identity. However, all hits were part of larger protein sequences such as the predicted secreted protein XP_002406990 from the black-legged deer tick Ixodes scapularis (Ixodida: Ixodidae), corroborating that the H-WG\(_{11}\)-NH\(_{2}\) peptide may originate from post translational processing.

Table 1. Proteins identified as present in the defence droplets of Z. filipendulae. In total, 66 different proteins were identified by LC-MS/MS. Defensive proteins such as protease inhibitors, proteases and oxidases were most abundant. MS and MS/MS tolerances were set to 10 ppm and 0.8 Da, respectively; false discovery rate set to 1%. The annotated functions are according to BLAST searches. Number in brackets indicates number of different proteins identified. For further details see Table S3 and supplementary methods.

<table>
<thead>
<tr>
<th>Protease inhibitors (18):</th>
<th>Hormone/odorant binding protein (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine protease inhibitor (17)</td>
<td>C-type lectin (3)</td>
</tr>
<tr>
<td>Trypsin inhibitor (1)</td>
<td>Aldo-keto reductase (2)</td>
</tr>
<tr>
<td>Proteases (10):</td>
<td>Peptidyl prolyl cis-trans isomerase (2)</td>
</tr>
<tr>
<td>Serine protease (7)</td>
<td>Phosphatidylethanolamine binding (1)</td>
</tr>
<tr>
<td>Metalloprotease (2)</td>
<td>Transferrin (1)</td>
</tr>
<tr>
<td>Cysteine protease (1)</td>
<td>Apolipoprotein D (1)</td>
</tr>
<tr>
<td>Oxidases (4):</td>
<td>Calcyphosine-like (1)</td>
</tr>
<tr>
<td>Prophenoloxidase (2)</td>
<td>Cuticular protein tweedle motif (1)</td>
</tr>
<tr>
<td>Peroxidase (1)</td>
<td>Split ends-like (1)</td>
</tr>
<tr>
<td>Sulfhydryl oxidase (1)</td>
<td>Calcyphosine-like (1)</td>
</tr>
<tr>
<td>Hydrolyases (8):</td>
<td>γ-interferon lysosomal thiol reductase (1)</td>
</tr>
<tr>
<td>Phosphatase (2)</td>
<td>Yellow-C (1)</td>
</tr>
<tr>
<td>Ribonuclease (1)</td>
<td>Yellow-D (1)</td>
</tr>
<tr>
<td>Lipase (1)</td>
<td>Ommochrome-binding protein-like (1)</td>
</tr>
<tr>
<td>β-glucosidase ZfBGD1 (1)</td>
<td>Flexin domain-containing protein (1)</td>
</tr>
<tr>
<td>α-amylase (1)</td>
<td>Chemosensory protein (1)</td>
</tr>
<tr>
<td>Chitinase-like (1)</td>
<td>Unknown (1)</td>
</tr>
<tr>
<td>α-trehalase (1)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Defence droplets glue together mouthparts, legs and sensory organs of potential predatory arthropods. (A) After physical irritation, Z. filipendulae larvae release viscous defence droplets which wet the hydrophobic cuticle of ants such as M. rubra (A’) including their mouthparts such as mandibles and sensory organs such as antennae. (B + B’) Water does not wet the hydrophobic cuticle neither of Z. filipendulae nor of the ant. (C + C’) A 0.1% SDS solution wets the cuticle of Z. filipendulae larvae as well as of ants. (D) Spiders such as M. acalypha may also induce the release of defence droplets, (D’) which glue their legs and immobilise them (arrow).
Scientific RepoRts | 6:22407 | DOI: 10.1038/srep22407

Figure 3. Mass spectrometric de novo sequencing of a major peptide from defence droplets. (A) MALDI-TOF-MS spectrum of defence droplets directly deposited on target. (B) MS/MS fragment spectrum of the MH⁺ 831.35 ion from the MS spectrum. Only annotated y- and major b-ions as well as immonium (im) ions are labelled in the spectrum for clarity. The sequence is shown with annotated fragment ions below the spectrum according to nomenclature of⁶¹. The C-terminal Gly is α-amidated. (C) Primary structure of the peptide.

Metabolite profiling of the defence droplets and haemolymph. To identify non-proteinaceous and small metabolites, an unbiased analysis of the metabolite composition of the defence droplets and haemolymph was carried out using GC-MS (Gas chromatography-mass spectrometry). An earlier comparison of the metabolite profile of the defence droplets and the haemolymph had shown that both defence droplets and haemolymph contain high amounts of the CNglcs linamarin and lotaustralin¹⁷. However, these CNglcs can only be detected by LC-MS and not by GC-MS due to their inherent thermal instability. In both defence droplets and haemolymph, the main metabolite components detected were carbohydrates and free amino acids (Table S1). Organic acids and sterols were present in both tissues in minor amounts, while a few higher alkanes and terpenes were detected in the haemolymph. Eighteen different carbohydrates were identified in the defence droplets of which the non-protein amino acid β-cyanoalanine was by far the most abundant one (1.0% relative abundance).

Testing β-glucosidase activity and HCN release from the defence droplets and haemolymph. Since Z. filipendulae larvae emit HCN³⁰,³¹, it was anticipated that the specific β-glucosidases required to hydrolyse linamarin and lotaustralin would be located in the released droplets. The only β-glucosidase detected in the defence droplets was ZfBGD1 (Table 1). A partial nucleotide sequence from ZfBGD1 was found in the Z. filipendulae larval transcriptome and a full-length sequence was obtained by RACE (Rapid amplification of cDNA ends)-PCR. Semi-quantitative RT (Reverse transcriptase)-PCR showed that ZfBGD1 was predominantly expressed in the larval integument, which contains the cuticular cavities harbouring the defence droplets (Fig. 4). To elucidate whether the ZfBGD1 enzyme was involved in CNGlcs-hydrolysis, it was expressed in Sf9 insect cells and purified. Recombinant ZfBGD1 was demonstrated to be functionally active by its ability to hydrolyse the generic β-glucoside substrate 4-methylumbelliferyl β-D-glucopyranoside (MUglc) at a rate of 17.8 nmol MU/h/μg ± 1.7 s.e.m. Surprisingly, ZfBGD1 did not show any hydrolytic activity towards linamarin and lotaustralin (0.0 nmol HCN/h/μg). In agreement with this result, the defence droplets exhibited β-glucosidase activity against MUglc (10.1 nmol MU/h/μg), but none against linamarin nor lotaustralin (0.0 nmol HCN/h/μg). This finding was supported by Raman spectroscopy, which demonstrated that the CNGlcs in the droplets stayed intact over time as monitored by the diagnostic and well-resolved absorption band near 2245 cm⁻¹. This finding was supported by Raman spectroscopy, which demonstrated that the CNGlcs in the droplets stayed intact over time as monitored by the diagnostic and well-resolved absorption band near 2245 cm⁻¹. The second most abundant carbohydrate was myo-inositol with a relative abundance of almost 1%. In the haemolymph, 15 different carbohydrates were detected with the major constituent being allose (22%) and trehalose (18.7%). Four amino acids were detected in the defence droplets of which the non-protein amino acid β-cyanoalanine was by far the most abundant one (1.0% relative abundance). Similar β-cyanoalanine content (0.9%) was found in the haemolymph, but the diversity of amino acids was higher in this tissue (Table S1).

Formation of crystalline-like precipitates in defence droplets. Around 5 minutes after release, the defence droplets began to harden, i.e. they changed from being highly viscous to sticky to finally solid. At this point the Z. filipendulae larvae were no longer able to reabsorb the droplets. The hardening process was completed within a circa 10 min period. The hardened structures were analysed in more detail via scanning electron and differential interference contrast microscopy. This revealed that the hardening of defence droplets resulted in the formation of largely insoluble crystalline-like precipitates (Fig. 5). The dimensions of the largest crystalline...
Figure 4. The β-glucosidase ZfBGD1 is specifically expressed in the integument. Semi-quantitative RT-PCR on ZfBGD1 using cDNA derived from different larval tissues of Z. filipendulae. Abbreviations: fb-fat body, gonads, gu-gut, lg-labial glands, Mt-Malpighian tubules, in-integument, ha-haemolymph. As positive controls 18S rRNA primers and whole larval cDNA (+) were used; water (−) as template constituted the negative control. Note that the integument contains cuticular cavities, which harbour the defence droplets.

Figure 5. Formation of crystalline-like precipitates in defence droplets after their release. Precipitates composed of particles with dimensions as large as 50 × 30 μm and irregular shapes ranging from almost oval (A) and triangular with sharp edges (B), as seen by electron microscopy, to crystal-like needle shapes (C,D) that are up to 50 μm long as seen via differential interference contrast microscopy. The scale bar equals 20 μm.

precipitates reached 50 × 30 μm with irregular shapes ranging from almost oval (Fig. 5A) and triangular with sharp edges (Fig. 5B) to needle-like crystals that were up to 50 μm long (Fig. 5C,D).

Water evaporation and build-up of antiparallel β-sheets during droplet hardening. The possible involvement of proteins and the importance of their secondary structures during hardening and precipitate formation in defence droplets were investigated using Fourier-transform infrared spectroscopy (FT-IR). The measurements documented that the amide I and amide II bands at 1622 cm⁻¹ and 1515 cm⁻¹, respectively, increased significantly around 5 minutes after defence droplet release when the hardening process started, and reached a maximum after 5 more minutes when the hardening process was completed (Fig. 6). Fresh defence droplets that were precipitated in 100% methanol showed similar characteristic amide I and II FT-IR bands at 1622 cm⁻¹ or 1521 cm⁻¹, respectively (Fig. S2). The increase of amide I and II band intensities at 1622 cm⁻¹ and 1515 cm⁻¹ indicates a strong build-up of antiparallel β-sheet structures33. The simultaneous decrease of the OH-stretching band at 3330 cm⁻¹ indicates loss of water concomitant to the hardening process of the defence droplets (Fig. 6). To determine whether these changes were directly related to the presence of the H-WG11-NH2 peptide, the peptide was obtained by chemical synthesis. FT-IR measurements using the synthesised peptide solubilized in distilled water did not result in build-up of antiparallel β-sheet structures. Instead, the peptide exhibited amide I and amide II bands at 1641 cm⁻¹ and 1553 cm⁻¹, respectively, which indicates a rather unordered structure33. Accordingly, the in vitro experiments do not indicate that the structural changes observed in defence droplets are directly linked to the presence of H-WG11-NH2. However, it is conceivable that other molecules or ions present in the droplets affect the structure of the peptide in vivo, possibly leading to formation of the antiparallel β-sheets.
Discussion

The chemical composition of defence secretions in arthropods and insects may be complex with the constituents packed into a viscous substance as reported for some species of centipedes and termites. In line with these previous studies, our studies on Z. filipendulae demonstrate versatile defence functions of droplets produced by a Lepidopteran larva. The first physical barrier a predator of Z. filipendulae larvae encounters is the high viscosity of the defence droplets, which serve to wet and glue together the hydrophobic cuticles such as of mandibles, legs and sensory organs as shown here for an ant and a spider (Fig. 2). Droplets of Z. filipendulae have surfactant-like properties, i.e. their surface tension is reduced in comparison to water. Similarly, the surfactant-like properties of oral secretions from the lepidopteran larva of Spodoptera exigua are able to wet the hydrophobic cuticle and to glue the antennae of ants, efficiently preventing further attacks. The ability of secretions to wet locomotory and sensory structures is highly effective as a defence system, since it may immobilise predators as well as impair proper sensory functioning. Viscous secretions may also serve to glue and block other susceptible structures like insect sensillae and spiracles needed for respiration.

Viscosity and stickiness of lepidopteran defensive secretions may be caused by the presence of proteins. Proteomic profiling of the Z. filipendulae defence droplets confirmed a highly proteinaceous content, which likely contributes to the viscosity and stickiness of the defence droplets. The glycine-rich peptide H-WG11-NH₂ in particular was highly abundant in the defence droplets (Fig. 3), but was absent from the non-viscous haemolymph. H-WG11-NH₂ may be an important functional component of the defence droplets because the amino acid glycine is characterised by intermediate hydrophobicity and thus able to form intermolecular interactions with water and other constituents in the droplets thereby contributing to protein solubility.

Given that free glucose constitutes almost 94% of all small metabolites detected in the defence droplets (Table S1), glucose may also contribute to the viscosity/stickiness of defence droplets in addition to the proteinaceous content. The polysaccharide dextran and its monomer glucose are known to decrease the surface tension of solutions, which may explain why Z. filipendulae larvae are able to wet and subsequently glue together the hydrophobic structures of predatory arthropods (Fig. 2). In contrast, in the non-viscous haemolymph, other sugars such as allose and trehalose were dominant, whereas glucose constituted a minor component. Trehalose, the non-reducing disaccharide of glucose, is often the main sugar in the haemolymph of insects, whereas the monosaccharide allose is a rare, possibly so far undetected component in insect haemolymph.

The largest groups of the proteins identified in the defence droplets were protease inhibitors, proteases and oxidases (prophenol oxidases, peroxidases) (Table 1). Because of their defensive properties, these proteins have been reported as key components employed by insects and plants to fend off diverse enemy species. Defence secretions of grasshoppers may contain a diversity of protease inhibitors directed against entomopathogenic fungi. Ingestion of plant tissue containing proteases may severely disrupt the peritrophic membrane in the insect midgut resulting in impaired nutrient utilization and thus reducing growth by 60–80%.
defence secretions in some Chrysolina leaf beetles may contain oxidases, which catalyse the generation of salicin-based feeding deterrents45. In a similar fashion, predators of Z. filipendulae may suffer damage from the defence proteins present in the defence droplets, if ingested.

β-Cyanoalanine was found in both the defence droplets and the haemolymph (Table S1); in the droplets it was the most abundant amino acid and the third most abundant small metabolite. β-Cyanoalanine is a detoxification product of HCN, catalysed by β-cyanoalanine synthases, and occurs in many lepidopteran species46,47. However, for vertebrate species β-cyanoalanine can also function as a neurotoxin48. Rats and chickens show signs of intoxication such as hyperirritability, tremors, convulsions and ultimately death only 13 h (rats) or 4 d (chickens) after being fed a diet containing 1.5% or 0.25% β-cyanoalanine, respectively49. Thus, apart from the toxicity residing in linamarin and lotaustralin, the β-cyanoalanine in the defence droplets of Zygaea may also be toxic.

The high content of CNglcs in the defence droplets and the finding that Z. filipendulae larvae release HCN50,51 intuitively suggested that a specific β-glucosidase should also be present in the defence droplets, perhaps only activated upon droplet release. The proteomic analysis revealed ZfBGD1 as the only β-glucosidase in the defence droplets (Table 1). ZfBGD1 expressed in insect cells catalysed hydrolysis of the generic β-glucosidase substrate MUglc, whereas it did not catalyse hydrolysis of the glucosidic bond in linamarin or lotaustralin. The same result was obtained when defence droplets were incubated with these substrates. Thus, the lack of HCN release from the defence droplets matches the inability of ZfBGD1 to hydrolyse CNglcs. Defence secretions that release HCN have been found in other Lepidopteran species such as thyridid caterpillars (Calindoea trifascialis)50, in Coleopteran species (Paropsida atomaria)51 and in species from other arthropod orders such as Chilopoda (Geophilus vitatus)52 and Diplopoda (e.g. Polydesmus complanatus)53. However, no specific β-glucosidase involved in CNglc catabolism has been characterised in these species yet.

Raman spectroscopy carried out on the defence droplets showed that the signal specific to the nitrile group of the CNglcs remained intact over time (Fig. S1). This provided independent support to the finding that the CNglcs present in the defence droplets were stable and not hydrolysed by ZfBGD1. The release of defence droplets by Z. trifolii has been shown to repel vertebrate predators such as starlings and lizards16. If the absence of a β-glucosidase that can hydrolyse CNglcs is a general phenomenon in the defence droplets of Zygaena species, the intact CNglcs may instead function as deterrents based on their bitter taste46,53. Secretion of bitter chemicals minimizes injuries and predation because they enable predators to assess palatability based on taste and to reject prey due to bitterness aversion53,54. In the rare case that Zygaena larvae are swallowed by predators, the CNglcs in the defence droplets may be hydrolysed into toxic HCN in the predator’s gut by digestive β-glucosidases, which are at least partly active towards linamarin or lotaustralin. Rats given a single oral dose of linamarin (500 mg/kg) or a lower oral dose of cyanide (6 mg/kg) showed signs of intoxication such as cardiac arrhythmias and respiratory changes indicating that the ingested linamarin was at least partly hydrolysed during passage through the rat’s gut55.

We have demonstrated that the defence droplets of Z. filipendulae do not release HCN per se. Therefore, the moderate amount of HCN released from Z. filipendulae larvae as reported in other studies50,51 may be generated in the haemolymph, which also contains high amounts of linamarin and lotaustralin (~11 μg per μl)17. In our study, Z. filipendulae haemolymph was found to release HCN as expected from a β-glucosidase catalysed reaction. Interestingly, an enzyme preparation from haemolymph devoid of CNglcs and spiked with defence droplets Z. filipendulae larvae resulted in a moderate amount of HCN released from the defence droplets and indicate that only a minor fraction of HCN produced in the larval haemolymph is released to the environment. Moreover, since many predators are expected to injure the larva, exuding haemolymph from a wound will most likely mix with defence droplets onto the cuticle resulting in the release of toxic and deterring HCN. Consequently, high amounts of HCN are produced: (i) outside of the larval body and (ii) only on demand, i.e. after attack and tissue damage and (iii), after mixing of haemolymph-located enzymes and droplet-based substrates.

When wetted by viscous secretions, the appendages of arthropod predators may become stuck together. To avoid this, some predators perform cleansing behaviour27. If the appendages remain stuck together the predator may die, especially when the secretion hardens after exposure to air as reported in this study and as previously observed with secretions from termites and centipedes53,54. The hardening process in the Z. filipendulae defence droplets starts approximately 5 minutes after droplet release, is completed after 5 more minutes and is accompanied by the formation of crystalline-like precipitates (Fig. 5). The hardened secretions may also act as physical deterrents based on their abrasive effects on predator’s mandibles and thereby interfere with digestion. Similarly, calcium oxalate crystals in many plant species have pronounced abrasive effects on mandibles of chewing caterpillars and ingestion of these crystals leads to reduced growth and increased mortality56.

Antiparallel β-sheets are abundant secondary structures in globular proteins and often involved in protein aggregation57. Formation of antiparallel β-sheets was observed in the defence droplets starting and finishing at the same time as the hardening process (Fig. 6). Enhanced protein concentrations as a result of water evaporation probably stimulated aggregation and final hardening of the defence droplets. In the silkworm Bombyx mori (Lepidoptera), the viscosity of the silk solution is increased by increasing the concentration of protein in the silk gland58. Similarly the viscous defence secretions of the African termite Odontotermes badius harden due to water evaporation, which may be accompanied by denaturation of endogenous proteins59. The finding that the amide I and II regions of the FT-IR spectra of defence droplets denatured with methanol were identical and similar, respectively, to the spectra obtained from the final state of hardened defence droplets (Fig. S2) strongly support that the hardening of the Z. filipendulae defence droplets is the combined result of protein aggregation, caused by formation of antiparallel β-sheets, and water evaporation.
This study illustrates that the defence droplets of *Z. filipendulae* larvae harbour a battery of versatile and composite physical and chemical defence systems to be deployed against predators, and provides insights into the underlying mechanisms of the defence. The defence droplets situated in cuticular cavities of the larvae can be regarded as morphological depositories, physically separated from the rest of the body. They enable the larvae to accumulate diverse defence components without the risk of self-intoxication, and at the same time, predators are immediately exposed to this defence weapon after attack.

**Methods**

**Predator assays.** Larvae of *Z. filipendulae* were collected from a natural population in the south-west of Taastrup (55.65°N, 12.30°E), Denmark. As examples of potential *Z. filipendulae* larvae predators, individuals of the common red ant *M. rubra* (Hymenoptera: Formicidae; six biological replicates) and the cricket-bat orb weaver spider *M. acalypha* (Aranae: Araneidae; two biological replicates) were collected from natural populations in Frederiksberg (55.68°N, 12.51°E) and Taastrup, Denmark. 0.5 μl of defence droplets from fifth instar *Z. filipendulae* larvae (out of seven instar stages), distilled water or 0.1% SDS was applied on the head and mandibles of the ants or on the cuticles of *Z. filipendulae* fifth larval instar. 2 μl defence droplets were applied to the legs of the spiders. The effects of the defence droplets were recorded using a digital camera (Nikon DS-Fi1) mounted on a light microscope (Leica Wild M3Z).

**LC-MS/MS.** To identify proteins present in the defence droplets, 1 μl fresh defence droplet from fifth instar *Z. filipendulae* larvae was digested with trypsin after denaturation, reduction and alkylation of Cys. An aliquot of the resulting proteolytic digest was desalted and subjected to nano-reverse-phase LC-MS/MS. Two biological replicates were analysed. The amino acid sequence data obtained were searched against a tran-...
In each case, three biological replicates were analysed and HCN release was measured as described elsewhere. Mean values with standard error were calculated and tested for significance using Student’s t-test (one-tailed, SigmaPlot 12).

Microscopy. 1 μl defence droplet freshly taken from a fifth instar of *Z. filipendulae* larva was air-dried on a microscope glass slide, placed on a carbon conductive adhesive tab mounted to an aluminum stub and sputter coated with a 1:1 gold-palladium mixture. Micrographs of the sample were taken using scanning electron microscopy at 10–15 kV on a Quanta 200 SEM (FEI Company). To analyse the inner structures of untreated and thus uncoated defence droplets, differential interference contrast microscopy was applied. Therefore, 1 μl fresh defence droplet was placed on a glass slide with immersion and then covered by a cover slip. After hardening and formation of crystalline-like structures, pictures were taken on a Leica DM 5000B fluorescence microscope using differential interference contrast. For each microscopy technique, two biological replicates were analysed.

Spectroscopy. Fourier-transform infrared spectra (FT-IR) were collected on an Arid-Zone MB100 FT-IR instrument (ABB Bomen, Quebec, Canada) using an attenuated total reflectance device with a triple-bounce diamond crystal. The IR spectra were recorded in the range from 4400–750 cm⁻¹ using a spectral resolution of four cm⁻¹. Droplets were freshly taken from fifth instar *Z. filipendulae* larvae. Test samples were either 2 μl of *i.*) defence droplets (three biological replicates), *ii.*) 250 mM H-WG₁₁-NH₂ (purity >90%, synthesized by JPT Peptide Technologies) diluted in distilled water (three technical replicates) or *iii.*) defence droplets diluted 1:10 in 100 % methanol from which the pellet, i.e. precipitate, was taken (three biological replicates). Spectra were recorded every 30 s with eight accumulations over a period of 15 min. Each spectrum represented the average of 32 scans compared to the background (64 scans) collected with the empty crystal and stored as absorbance spectra. CNglcs can be specifitically measured by Raman spectroscopy due to the nitrile group, which has a well-resolved CN stretching band near 2245 cm⁻¹. Raman spectra were collected on a RamanRxn1 instrument equipped with an Invictus™ 785 nm near-infrared diode laser (Kaiser Optical Systems Inc., MI, USA). Data were collected using a single holographic grating and a thermoelcctro cooled charge-coupled device detector, operated at −40 °C. The probe was an Mk II filtered probe attached to a Leica Raman microscope equipped with a 10 x objective. The Raman spectra of 2 μl defence droplets (three biological replicates) were recorded every 30 s with eight accumulations of 1 s exposure for a total period of 15 min. The spectra were stored as Raman shifts in the range 3500–0 cm⁻¹.

References


**Release of HCN.** For analysis of general β-glucosidase activity in the defence droplets derived from fifth instar larvae, 1 μl droplet was incubated at 30 °C for 1 h in 240 μl 60 mM citric acid buffer (pH 6) containing 200 μM MUglc. Release of the aglucone MU was quantified as described above. To determine whether the β-glucosidase from the defence droplets could hydrolyse the CNglcs present in the defence droplets in vivo, 1 μl defence droplet was either incubated alone or together with 1 μl enzyme preparation purified from larval haemolymph of the same instar and devoid of CNglcs as positive control at 30 °C for 1 h in 200 μl 60 mM citric acid buffer (pH 6). For comparison to HCN emission from the haemolymph, 1 μl crude haemolymph was incubated with 1 μl haemolymph devoid of CNglcs at the same conditions. In each case, three biological replicates were analysed and HCN release was measured as described elsewhere. Mean values with standard error were calculated and tested for significance using Student’s t-test (one-tailed, SigmaPlot 12).

Acknowledgements

We thank M. Saddik Motawia for providing chemically synthesized lotaustralin. Joel Fürstenberg-Hägg is thanked for RNA extraction of some Z. filipendulae tissues. We thank Shengjun Wang for assistance with respect to purification of recombinant ZfBGD1. We also acknowledge Jørgen Lissner for identifying M. acalypha and we thank Catherine A. Skrzynski Nielsen for assistance with differential interference contrast microscopy. We thank Ros Gleadow for proof-reading and helpful comments on the manuscript. Imaging data were collected at the Center for Advanced Bioimaging (CAB) Denmark, University of Copenhagen. Financial support from the following institutions is highly acknowledged: VILLUM Research Center “Pro-active Plants” and “Plant Plasticity”, The Danish Council for Independent Research (grant # DFF – 1323-00088), The Danish National Research Foundation (grant DNRF107), Copenhagen University Excellence Programme for Interdisciplinary Research (CDO2016) and The Danish Councils for Strategic and Independent Research (12-131859).

Author Contributions

Conceived and designed the experiments: S.P., M.Z., S.B.E., B.L.M. and S.B. Performed the experiments: S.P., M.Z., B.K., S.B.E., H.C., B.L.P. and J.B. Analysed the data: S.P., M.Z., B.K., S.B.E., H.C., B.L.P. and J.B. Wrote the paper: S.P., M.Z., S.B. and B.L.M.

Additional Information

Accession code: ZfBGD1 (ENA accession number: LN851681).

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pentzold, S. et al. Lepidopteran defence droplets - a composite physical and chemical weapon against potential predators. Sci. Rep. 6, 22407; doi: 10.1038/srep22407 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/