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RESEARCH ARTICLE

Too hard to swallow: a secret secondary defence of an aposematic insect

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ABSTRACT

Anti-predator strategies are significant components of adaptation in prey species. Aposematic prey are expected to possess effective defences that have evolved simultaneously with their warning colours. This study tested the hypothesis of the defensive function and ecological significance of the hard body in aposematic *Pachyrhynchus* weevils pioneered by Alfred Russel Wallace nearly 150 years ago. We used predation trials with *Japalura* tree lizards to assess the survivorship of 'hard' (mature) versus 'soft' (teneral) and 'clawed' (intact) versus 'clawless' (surgically removed) weevils. The ecological significance of the weevil's hard body was evaluated by assessing the hardness of the weevils, the local prey insects, and the bite forces of the lizard populations. The existence of toxins or deterrents in the weevil was examined by gas chromatography-mass spectrometry (GC-MS). All 'hard' weevils were instantly spat out after being bitten once and survived attacks by the lizards. In contrast, the 'soft' weevils were chewed and subsequently swallowed. The results were the same regardless of the presence or absence of the weevil's tarsal claws. The hardness of 'hard' *Pachyrhynchus* weevils was significantly higher than the average hardness of other prey insects in the same habitat and the mean bite forces of the local lizards. The four candidate compounds of the weevil identified by GC-MS had no known toxic or repellent functions against vertebrates. These results reveal that the hardness of aposematic prey functions as an effective secondary defence, and they provide a framework for understanding the spatio-temporal interactions between vertebrate predators and aposematic insect prey.

KEY WORDS: Anti-predator strategy, Aposematism, Hardness, *Japalura* lizard, *Pachyrhynchus* weevil, Taiwan

INTRODUCTION

'… *tropical weevils which have the elytra and the whole covering of the body so hard as to be a great annoyance to the entomologist, …, they cannot be pinned without first drilling a hole to receive the pin, and it is probable that all such find a protection in this excessive hardness*.'

'*The insects which others imitate always have a special protection, which leads them to be avoided as dangerous or uneatable by small insectivorous animals; some have a disgusting taste …; others have such a hard and stony covering that they cannot be crushed or digested;…*'

Alfred Russel Wallace, 1867

Predation is one of the most visible selective forces driving the ecology and evolution of organisms in nature (Abrams, 2000). Therefore, evolving effective anti-predator strategies is a significant component of adaptation for many prey species. Diverse defensive strategies have evolved in a range of specific stages in the encounters between predators and preys (Stevens, 2013). These anti-predator strategies can be classified as 'primary' and 'secondary' defences, depending on the timing in which they are performed (Ruxton et al., 2004). The primary defences serve to avoid detection (crypsis) by operating before predators initiate prey-catching behaviour, or they prevent pursuit by advertising themselves as unprofitable to predators (mimicry, warning signals) (Robinson, 1969; Edmunds, 1974). The secondary defences are post-detection defences that function to increase the chance of surviving the prey-capturing process (deflection or startle) or to make an encounter unprofitable to predators (e.g. spines, stings and toxins) (Robinson, 1969; Edmunds, 1974). The development of the secondary defences that include chemical or physical deterrents has led to the evolution of warning signals in prey that benefit both well-defended prey and their potential predators by causing mutual avoidance (Summers and Clough, 2001; Sherratt and Beatty, 2003; Ruxton et al., 2004). Well-known examples of co-evolution between warning signals and prey defences include the generation of aposematic colouration and toxicity in poison frogs (Summers and Clough, 2001; Maan and Cummings, 2012) and ladybird beetles (Blount et al., 2012).

Pachyrhynchus weevils (Germar 1824) (Coleoptera: Curculionidae: Entiminae: Pachyrhynchini) are a group of brilliant, metallic-coloured weevils distributed in the Old World tropics (Fig. 1A) (Wallace, 1895; Schultze, 1923; Tseng et al., 2018). The observations of the diverse colours of more abundant *Pachyrhynchus* weevils (as models) being mimicked by several relatively rare species of longhorn beetles (Cerambycidae: *Doliops*) suggested the aposematic function of their colouration (Wallace, 1867; Dickerson et al., 1928; Barševskis, 2013). However, *Pachyrhynchus* weevils may also be mimicking *Doliops* because many longhorn beetles are known to be toxic (reviewed in Dettner, 1987). A recent manipulative experiment showed the first empirical evidence that the conspicuous colouration of *Pachyrhynchus* weevils could function as effective warning signals (a primary defence) to prevent predacious pursuit by *Japalura* lizards (Fig. 1C) (Tseng et al., 2014). In those behavioural trials, the lizards attacked weevils without conspicuous colours at higher rates than weevils with intact colours. During those trials, the lizards were observed to display irritated behavioural responses (spitting out and throwing off) after biting the weevils, indicating the unpalatability of the prey.

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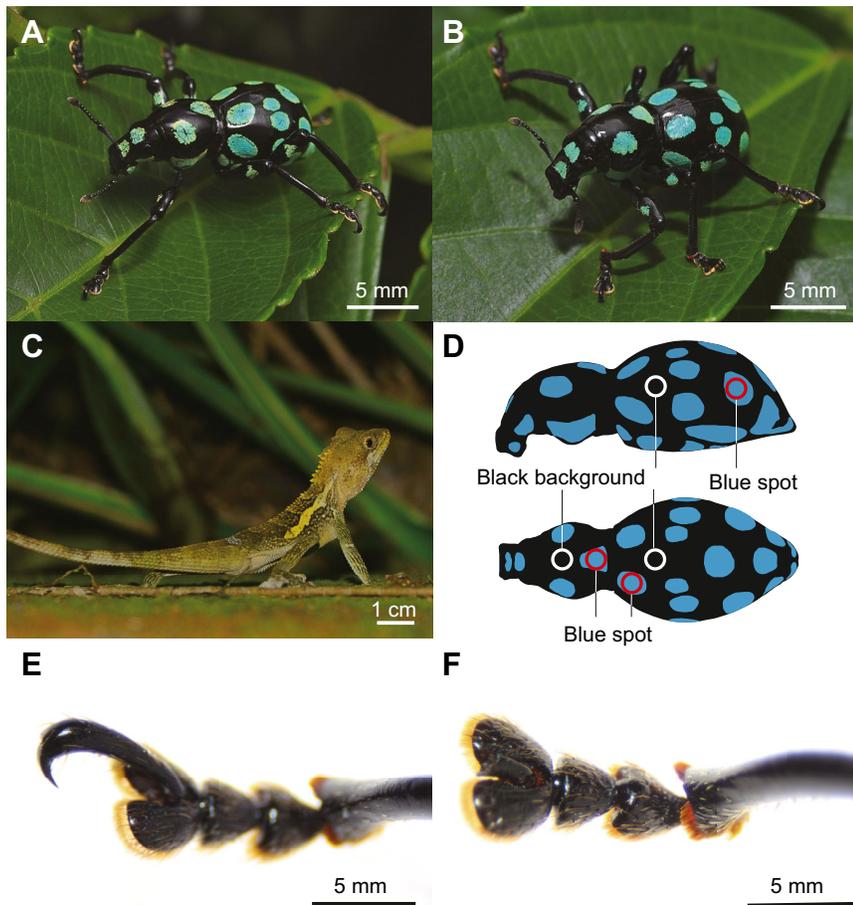
Fig. 1. *Pachyrhynchus sarcitis kotoensis* and experimental manipulation. (A) 'Hard' and (B) 'soft' *P. sarcitis kotoensis*. (C) The predator, *Japalura swinhonis* lizard. (D) The locality for measuring the colour spectrum of the blue spots (red circles) and the black background (white circles) of *P. sarcitis kotoensis*. (E) 'Clawed' (intact) and (F) 'clawless' (surgically removed) tarsi of *P. sarcitis kotoensis*. Images by L.-Y. Wang (A and B) and F. C. Hsu (C).

Moreover, the weevils frequently survived the lizard attacks without visible harm to their bodies, suggesting the existence of an effective secondary defence, which is expected to have evolved in concert with their warning colouration.

Alfred Russel Wallace first hypothesized that the excessive hardness of aposematic *Pachyrhynchus* weevils served as a defence mechanism against small insectivorous animals (i.e. the morphological defence hypothesis) (Wallace, 1867). Despite the fact that the defensive function appears to be a charming and conceivable explanation for these weevils with completely fused and rigid elytra, approximately 150 years after Wallace's proposal, we still do not understand the ecological and adaptive significance of the hard body of these weevils, and we have no solid empirical evidence to evaluate this hypothesis. Nevertheless, our earlier observations of only extremely large lizards (thus with higher biting force; ~3% of the trials) consuming the weevils pointed to the feasibility of Wallace's hypothesis (Tseng et al., 2014). Alternatively, other external rigid morphologies, such as sharp claws and spines on an insect's legs, can often be used as effective weapons against small vertebrate predators after being swallowed (Ruxton et al., 2004; Eisner et al., 2005). *Pachyrhynchus* weevils have three pairs of elongated legs that can strongly grip objects (Fig. 1E) (Schultze, 1923; Starr and Wang, 1992). Combined with the strong grip, the sharp tarsal claws at the tips of these legs could potentially cause the irritated behavioural response of the lizards, which have soft tongues and mouth cavities.

In addition to morphological (mechanical) defences, the secondary defences of animals can often take the form of toxic or distasteful chemicals (i.e. the chemical defence hypothesis) (Ruxton

et al., 2004). Although chemical defences are the most common secondary defensive strategies in aposematic prey, to date, no specialized secretory glands or storage sites for toxic chemicals have been found in *Pachyrhynchus* weevils (Pasteels et al., 1983). Therefore, these weevils are less likely to be chemically defended. However, a few *Pachyrhynchus* weevils are known to feed on poisonous plants (e.g. sea poison tree, *Barringtonia asiatica*, Lecythidaceae in *P. sonani*) (Chen et al., 2017); thus, their bodies may potentially contain distasteful chemicals or plant-derived toxins.

This study investigated the secondary defensive functions and ecological significance of the hard body of *Pachyrhynchus* weevils. First, the morphological defence hypothesis was tested using manipulative experiments that included lizard predation, which compared the survivorship between the 'hard' (mature) and 'soft' (teneral) weevils (Fig. 1A,B) and between the 'clawed' (intact) and 'clawless' (surgically removed) weevils (Fig. 1E,F). If the weevils used their hard bodies or tarsal claws as secondary defences against the lizards, the prediction was that after the encounters with lizards, either the 'hard' weevils would have a higher survival rate than the 'soft' ones, or the 'clawed' weevils would survive better than their 'clawless' counterparts. Next, the ecological relevance and adaptive significance of the weevil's hard body was assessed by measuring and comparing the hardness of the weevils and other insect prey species found in the lizard's natural habitat. Because the bite capacity is an important functional trait and ecological indicator for the width of the diet niche of the lizards (Herrel et al., 2001; Meyers and Irschick, 2015), the hardness (measured as force at failure) was compared with the bite forces of the local lizard populations. If the

hard bodies of the weevils represented an important adaptive defence mechanism within the potential prey spectrum of the lizards, the prediction was that the hardness of the weevils would be higher than that of the other potential prey in the lizard's habitat. The hardness of the weevils was also expected to be higher than the average bite force of the local lizard populations. Finally, the chemical defence hypothesis was tested by analysing the biochemical content of the weevils for the existence of toxins or deterrents of the predator vertebrates. We predicted to find no toxic or distasteful chemicals in the weevils.

MATERIALS AND METHODS
Ethics statement
The permission to use the protected *Pachyrhynchus* weevils was granted by the Forestry Bureau, Council of Agriculture, Taiwan (permit numbers 1031700770, 1041700842 and 1060241435). The Institutional Animal Care and Use Committee of the National Taiwan Normal University approved the behavioural experiments using *Japalura* lizards (number 105012). The animal ethics protocols of the Wildlife Conservation Act of Taiwan were followed throughout the experiments, and no harmful effects to the lizards were observed. All lizards were released to their capture locations after the experiments, and the weevils were fed and kept in the laboratory until they died.

Weevils and lizards
Six adults (three males and three females) of the endemic Taiwanese *Pachyrhynchus sarcitis kotoensis* Kôno 1930 (Fig. 1A) were collected by hand from Orchid Island in August 2015. This *Pachyrhynchus* species was chosen for the study because it is relatively large and easy to rear in the laboratory. The collected weevils were reared individually in cylindrical plastic containers (8 cm diameter, 6 cm height) in the laboratory at 25°C and under 12 h:12 h light:dark cycles. Once a week, they were supplied with fresh leaves of the known host plant, *Leea guineensis* (Leeaceae). These weevils were later placed together in the containers and allowed to mate freely. The eggs and the first instar larvae produced by these individuals were reared inside the stems of their host plants. The plant stems were kept individually in transparent plastic boxes (5.4×4.3×4.0 cm) at 25°C with 12 h:12 h light:dark cycles. Once eclosed, the teneral adults stayed in their pupal chambers for approximately 5 days. The cuticles of the teneral adults remained soft (unsclerotized) for at least 7 days after they emerged from the chambers. For the following behavioural trials, the 'soft' weevils were defined as the individuals still within the first 5 days of emergence from the pupal chambers (Fig. 1B); the 'hard' weevils were defined as those having been emerged for more than 2 months (Fig. 1A). The 'clawless' weevils (Fig. 1F) were made by removing all of their tarsal claws with scissors.

The diurnal tree lizards, *Japalura swinhonis* (Agamidae) (Fig. 1C), are common arboreal predators in lowland forests of Taiwan and the Orchid and Green Islands. They are sit-and-wait predators that use mainly visual cues for prey detection, and they feed on arthropods, including weevils (Huang, 2007). Therefore, *J. swinhonis* was selected as the testing predator for *Pachyrhynchus* weevils in this study. Seventy-eight adult lizards [males: N=60, snout–vent length (SVL) 73.16±4.79 mm; females: N=18, SVL 66.57±4.13 mm] were collected using a noose between May and October of 2016 in the forests of Taipei Zoo (24°59′36.4″N, 121°34′51.9″E). The allopatrically distributed (thus probably naive) Taipei lizard population was used to increase the attacking rate on weevils in the behavioural trials because the sympatric lizards attack

weevils at lower frequencies (Tseng et al., 2014). However, the body sizes of Taipei lizards are smaller than those of the Green Island (SVL, males: 76.59±4.44 mm, N=92; females: 63.40± 5.09 mm, N=80) and Orchid Island populations (SVL, males: 75.93±4.87 mm, N=80; females: 69.60±4.06 mm, N=89), which were used for measuring bite forces (L.-Y.W., H.-Y. Tseng, J.-Y. Hsu and W.-S.H., unpublished data). For 3 days before the experiments, the captured lizards were kept individually in plastic containers (34×17×24 cm) at 25–30°C with water *ad libitum* in the laboratory. A 3-day period of food deprivation prior to the behavioural trials was used to increase the level of hunger in the lizards. Each lizard was used only once in the behavioural trials. The fifth toe on the right hindlegs of the lizards was clipped and used as a marker before releasing to prevent that individual's recapture.

Colour and mobility of 'hard' and 'soft' weevils
Before conducting the behavioural trials, we measured the colouration and daily rhythm of 'hard' and 'soft' weevils to examine their level of resemblance in appearance and mobility. Reflectance spectra of the colouration were measured with a spectrometer (detection range: 250–800 nm, Jaz spectrometer, Ocean Optics, Dunedin, FL, USA) connected to a reflection probe (ZFQ-13101) and illuminated by a deuterium–tungsten halogen light source (DH-2000-BAL). The spectrometer was calibrated with a reflectance standard (WS-1-SL). For each individual, we measured their three blue spots (Fig. 1D, red circles) and three black background areas (Fig. 1D, white circles). The light source was positioned perpendicularly 1 mm above the focal area of the weevil body surface (diameter of the illuminated area: ∼2 mm). The three measurements of the blue spots and black areas from each individual were averaged. The hue (wavelength of the apex of the spectrum), brightness (reflectance ratio of the apex of the spectrum), and saturation (area under the spectral curve between the visible wavelengths of diurnal lizards, 440–625 nm; Yewers et al., 2015) of the measured spectrum were calculated. The ultraviolet-sensitive (UVS) (364–383 nm) spectrum was excluded from calculation because of the low UV reflectance (<40%) of the colouration (Fig. 2A). No significant difference between the colour of 'hard' and 'soft' weevils was detected (Fig. 2A) (two-sample *t*-test, N=4; hue, 'hard': 555.36±19.76 nm; 'soft': 547.15±8.73 nm; t=0.76, P=0.476; brightness, 'hard': 128.97±4.79%, 'soft': 125.00± 3.99%, t=1.27, P=0.250; saturation, 'hard': 66.40±2.99%, 'soft': 67.23±1.90%, t=−0.45, P=0.666).

The daily rhythm of the weevils (N: 'hard'=10, 'soft'=10) was recorded at 25°C for six consecutive days using a video monitor (DS-VR7160H, Der Shuenn, Taipei, Taiwan). The weevils were fed once on the third day. Each weevil was placed in a Petri dish (diameter: 90 mm, height: 15 mm) divided into four equal quadrants. We recorded the number of weevil movements between the quadrants in the first 10 min of each 2-h increment. The total number of movements for each individual during the 6 days was summed as a proxy of its mobility. The daily rhythm of the two weevil groups was similar (Fig. 2B) (number of movements per day, 'hard': 42.5±15.9, 'soft': 44.6±15.9; two-sample *t*-test of daytime scores, N=10, t=−0.38, P=0.711; Mann–Whitney U-test of total scores, N=10, U=2097.5, P=0.048). These results suggested that the 'hard' and 'soft' weevils represented prey items of equal appearance and mobility for the visually oriented lizard predator.
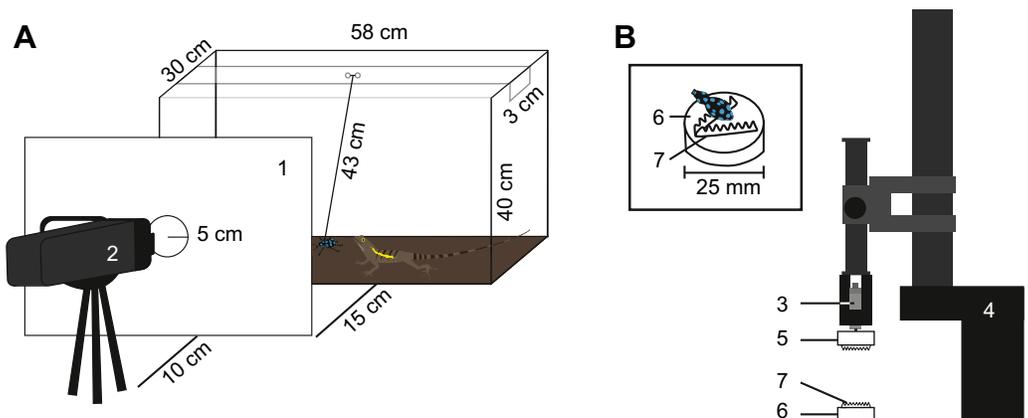
Behavioural trials
To test the effectiveness of the weevils' hard exoskeletons and tarsal claws against predation by lizards, the weevils were randomly divided
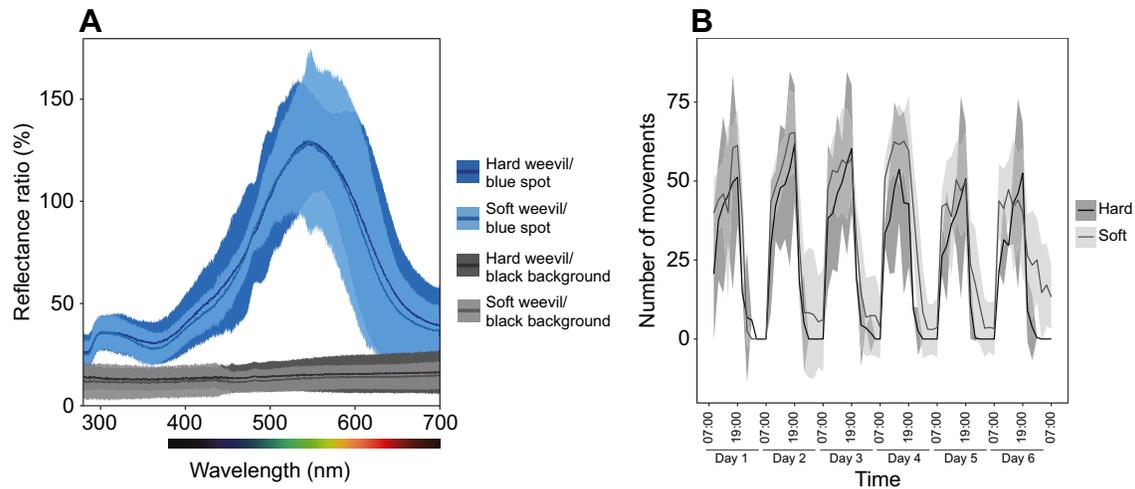
Fig. 2. The reflectance spectra and daily rhythm of 'hard' and 'soft' *P. sarcitis kotoensis* in the behavioural experiments. (A) The reflectance spectra of blue spot and dark background colours on the cuticles of the weevils ('hard' *N*=4, 'soft' *N*=4) were measured using a spectrometer (Jaz spectrometer, Ocean Optics). The coloured and grey shaded regions are the 95% confidence intervals of the measurements. (B) The daily rhythm of the weevils ('hard' *N*=10, 'soft' *N*=10) were recorded for six consecutive days using a video monitor (DS-VR7160H, Der Shuenn). The weevils were placed in Petri dishes divided into four equal quadrants. The number of the weevil's movements between the quadrants in the first 10 min of every 2 h was calculated. The shaded regions are the standard deviation of the number of movements.

into four treatments (*N*=78): (1) 'hard' weevils with claws, (2) 'hard' weevils without claws, (3) 'soft' weevils with claws, and (4) 'soft' weevils without claws. The trial arena was a transparent plastic container (58×30×40 cm) with its bottom covered with soil (Fig. 3A). The three vertical planes of the container were covered with light brown cardboard, and one side was left transparent for recording with a camcorder (HDR-XR200, Sony, Tokyo, Japan). The operator and the camcorder were located behind cardboard (75×100 cm) with an opening (diameter, 10 cm) to minimize disturbance. The trials were carried out between 10.00 and 18.00 h and at 26–30°C, which were the conditions when the lizards were most active. The lizards were placed into the arena 3 min prior to the trials for acclimation. The lizards appeared to settle down within 1 min of introduction; this was determined once they stopped running around or trying to climb out of the container. The weevils were tied with a black cotton thread, and the opposite side of the thread was tied to the centre of a transparent plastic bar (58×3×0.3 cm). The length of the thread was ~43 cm to confine the weevil's movement to the centre of the arena for video recording. Three minutes after placing the lizard in the arena, the weevil was introduced into the arena by hanging the plastic bar above the arena. The weevil was placed at the centre of the arena (approximately 30 cm diameter) and approximately 15–30 cm away from the lizard, which often positioned itself at the periphery. The trial started when the lizard showed awareness of the weevil by turning its eyes or head towards the prey and ended after the lizard stopped responding to the prey. The survival rate, number of bites, and prey handling time were calculated from the video footage.

Fig. 3. The set-up for behavioural trials of predation by *Japalura swinhonis* on *P. sarcitis kotoensis* and the measurement of hardness. (A) The bottom of the plastic container was covered with soil. The three inner vertical planes of the plastic container were covered with cardboard; one side was left transparent for video recording. The cardboard (1) was used to hide the operator and video camera (2) to avoid disturbing the lizards. The weevil was introduced to the arena using a black cotton thread tied between its thorax and abdomen, and attached to the centre of a transparent plastic bar. (B) Experimental set-up for measuring the hardness of insect prey. The force transducer (3) was attached to the lower end of the camera mount on the copy stand (4). The upper and lower jaws of the lizard were embedded in resin platforms (5 and 6). The upper jaw was attached to the force transducer and the lower jaw was placed directly below the upper jaw to match the upper one. The prey was oriented perpendicularly with respect to the lower tooth rows (7).

Insect prey and hardness measurement

To assess the level and distribution of hardness in the lizard's prey community, the insects occurring within the habitats of lizards were sampled by hand, with sweeping nets, light traps and pitfalls on Orchid and Green Islands in September and October 2015. The insects were collected along the forest trails where most lizards could be found (eight sites on Orchid Island for 5 days and four sites on Green Island for 4 days). The light traps (high-intensity discharge lamps, 35 W, 6000 K, Knight, Guangdong, China) were set up on Orchid Island for 2 h per night. The lepidopterans and spiders were not sampled because they are considered to be 'soft' prey (Verwaijen et al., 2002). One to two individuals of each insect species were obtained and later stored in a freezer at −20°C in the laboratory until the measurements were taken. All voucher insect specimens of this study were archived in the insect collection of the laboratory (Table S1).

The hardness of the insects (both field-collected insect prey and the 'hard' and 'soft' weevils) was measured following Herrel et al. (2001). The 'force at failure' of the elytra (abdomen) or the thorax of the insects was measured to represent the 'hardness' of the subjects. The 'failure' was defined as the complete loss of load-carrying capacity of the structure (Collins, 1993). The definition of 'hardness' in biological studies is debatable, and this term often refers to the phenomena caused by the combined effects of material and structural properties (e.g. for alternative 'intractability' in Evans and Sanson, 2005). Here, we use 'hardness' to indicate the maximum force at failure for crushing an insect's exoskeleton. The upper and lower jaws of the lizard were dissected, cleaned and then embedded in resin (model 40200029, Struers, Ballerup, Denmark; diameter: 25 mm) with the rows of teeth exposed (H in Fig. 3B) to build the biting platform (F and G in Fig. 3B). The upper part of the platform (F in Fig. 3B) was connected to a force transducer (model 9203, Kistler Inc., Wintherthur, Switzerland) (D in Fig. 3B), which was attached to the lower end of a camera mount on a copy stand (E in Fig. 3B) and connected to the charge amplifier (model 5995). The lower part of the platform was placed below the upper part in a position where the two jaws lined up completely. These insect specimens were measured at 25°C after they were fully thawed in the laboratory. Each specimen was positioned perpendicularly with respect to the rows of teeth. The upper platform was slowly moved down until the exoskeleton of the insect was broken or crushed, and the amount of force exerted on the transducer was recorded simultaneously. The breakdown of the exoskeleton of the specimen was recognized based on the structural failure of the exoskeleton. The maximum force needed to break the specimen's exoskeleton was recorded as a proxy for the hardness of the insect. These measurements were compared with the bite forces of the *J. swinhonis* populations from the Orchid and Green Islands (L.-Y.W., H.-Y. Tseng, J.-Y. Hsu and W.-S.H., unpublished data).

Chemical analysis

The presence of potential deterrent chemicals within the body of *P. sarcitis kotoensis* was examined using a gas chromatograph-mass spectrometer (GC-MS). The thorax and abdomen of three reared *P. sarcitis kotoensis* adults were separated and immersed in 3 ml of ether for 10 min of chemical extraction. From this extraction, 0.5 µl was used for the GC-MS analysis. The GC-MS system consisted of a gas chromatograph (model: TRACE 1300), a mass-selective detector (model: ISQ, Thermo Fisher Scientific, Waltham, MA, USA) and a GC column (inside diameter: 0.25 mm, length: 30 cm, film thickness: 0.25 µm) (model: Rxi-5 MS, 13423, Restek Corporation, Bellefonte, PA, USA). The inlet temperature was

280°C, and helium was the carrier gas with a constant flow of 1 ml min^{-1}. The initial temperature was 50°C and rose at a rate of 10°C min^{-1} to 300°C. This temperature was held for 10 min without delay of the solvent. The peaks with higher relative abundance were identified and compared with the spectrum libraries (NIST, Gaithersburg, MD, USA, and WILEY 10R, Hoboken, NJ, USA). If the SI (similarity index) and RSI (reverse similarity index) values of the candidate were both higher than 800, it was considered as a possible chemical compound extracted from the weevils (Tian et al., 2014). Finally, this compound was identified and it was determined if it represented a potential repellent for vertebrate predators with the online Pherobase (www.pherobase.net/).

Statistical analyses

The two-sample *t*-tests were used to compare the reflectance spectra and daily rhythm (daytime) between 'hard' and 'soft' weevils, and a Mann–Whitney *U*-test was used to compare the daily rhythm of day and night-time. Fisher's exact test and negative binomial regression were employed to compare the survival rate and number of bites, respectively, in the behavioural trials. In negative binomial regression and survival analysis, we used the elytra length of the weevils and the SVL of the lizards as covariates. These statistics were followed by Tukey's *post hoc* tests to examine the differences between the treatment groups. A Welch two-sample *t*-test was used to compare the hardness between 'hard' and 'soft' weevils, and linear regression analysis was conducted to assess the relationship between prey hardness and body size. All statistical analyses were carried out in R 3.3.1 (R Development Core Team, 2016).

RESULTS

Effects of the hardness and claws

Immediately after introducing the weevils into the trial arena, the lizards perceived the presence of the prey by turning their eyes and heads towards the weevils (Movies 1 and 2). Then the lizards approached and bit the weevils (often in the abdomen) using their lateral rows of teeth. After their introduction, the weevils were attacked by the lizards within an average of 18.8 s (18.8±22.9 s, *N*=59). Approximately half of the lizards (51%, *N*=76) showed no predatory response towards the weevils after more than 3 min, suggesting that they either were not interested, experienced neophobia, or avoided attacking aposematic prey items. After the lizard's first attack, the weevils were either spat out (Movie 1, 00:50) or consumed (Movie 2, 00:40) by the lizards. All 'hard' weevils were spat out immediately by the lizards, and the weevils survived the first attack. No rejected weevils had visible physical damage to the body, and they lived for more than 2 months after the trials (survival rate 100%, *N*=40) (Fig. 4A), except for two individuals that were injured and suffered a small depression on the surface of their lateral abdomen. The lizards made no more predatory attempts after spitting out the weevils (Movie 1). In contrast, all 'soft' weevils were chewed continuously and subsequently swallowed by the lizards (Movie 2, Fig. 4A) (survival rate 0%; Fisher's exact test: *N*=38; 'hard' with claws versus 'soft' with claws: *P*<0.001; 'hard' without claws versus 'soft' with claws: *P*<0.001; 'hard' with claws versus 'soft' without claws: *P*<0.001; and 'hard' without claws versus 'soft' without claws: *P*<0.001). The results were the same regardless of tarsal claws (Fig. 4A) ('hard' with claws versus 'hard' without claws: *P*=1; and 'soft' with claws versus 'soft' without claws: *P*=1).

The lizards bit 'soft' weevils significantly more times than they bit 'hard' weevils (Fig. 4B) (mean number of bites=1.5±1.0 for 'hard', 80.3±34.0 for 'soft'; negative binomial regression: χ^2=227.83, *P*<0.001) ('hard' with claws versus 'soft' with claws:
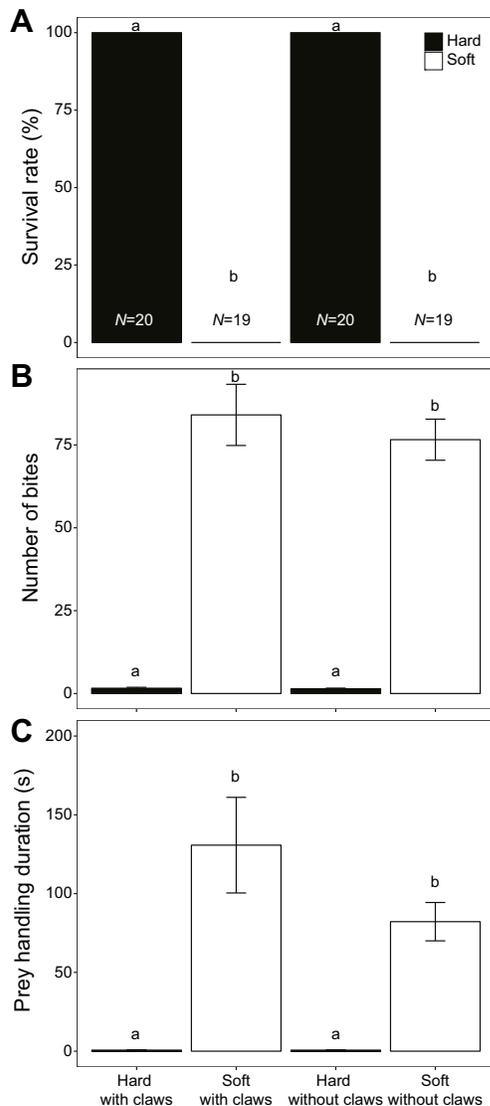
Fig. 4. The survivorship of the weevil *P. sarcitis kotoensis* and predatory response of the tree lizard *J. swinhonis* in the behavioural trials. The 'hard' treatments were significantly different from 'soft' treatments in (A) survival rate, (B) number of bites and (C) prey handling duration of the lizards. 'a' and 'b' indicate groups with significant differences (Tukey's *post hoc* tests, $P<0.001$).

$Z=18.99$, $P<0.001$; 'hard' without claws versus 'soft' with claws: $Z=-19.01$, $P<0.001$; 'hard' with claws versus 'soft' without claws: $Z=19.15$, $P<0.001$; and 'hard' without claws versus 'soft' without claws: $Z=19.04$, $P<0.001$), with no observed effect of tarsal claws ('hard' with claws versus 'hard' without claws: $Z=-0.48$, $P=1$; and 'soft' with claws versus 'soft' without claws: $Z=-0.06$, $P=1$). The lizards spent significantly more time handling 'soft' weevil prey than 'hard' weevil prey (Fig. 4C) (mean handling time=0.7 ± 0.5 s 'hard', 106.5 ± 102.4 s 'soft').

Hardness of prey and lizard bite force

The prey hardness increased allometrically with size for most prey species measured in the habitats (Fig. 5) [$\log_{10}$ (force at failure)= $-0.9389+1.5885 \log_{10}$ (body length), adjusted $R^2=0.53$, $P<0.001$, Akaike's information criterion (AIC)=69.95; force at failure= $-9.2195+1.5678$ body length, adjusted $R^2=0.65$, $P<0.001$, AIC=503.22], with the exception of grasshoppers (Acr,

Acrididae), owlflies (Asc, Ascalaphidae), dragonflies (Lib, Libellulidae) and katydids (Tet, Tettigoniidae), which were larger but softer insect prey. The 'hard' and 'soft' weevils deviated from the regression of hardness versus body length by being harder and softer, respectively, than other prey with similar sizes (Fig. 5). The 'hard' *P. sarcitis kotoensis* (32.6 ± 9.1 N, $N=17$) were significantly harder than their 'soft' counterparts (0.6 ± 0.6 N, $N=10$) (Welch two-sample *t*-test, $t=14.46$, $P<0.001$) and the average hardness of insect prey in the same habitat (Fig. 5) (9.95 ± 11.98 N, $N=72$), with the exception of two rhinoceros beetles, *Xylotrupes philippinensis* (93.5 and 60.8 N; black arrows 1 and 2, Fig. 5). The 'hard' *P. sarcitis kotoensis* were also harder than most other weevil species (Cur) in the habitat, except *Aclees hirayamai* (black arrow 3, Fig. 5). The average hardness value of 'hard' *P. sarcitis kotoensis* was higher than the mean bite force of *J. swinhonis* from Green Island (males: 27.97 ± 9.55 N, $N=91$; females: 8.14 ± 2.42 N, $N=80$) and Orchid Island (males: 29.66 ± 9.62 N, $N=80$; females: 12.03 ± 2.80 N, $N=89$) (Fig. 5), indicating that most lizards from these two islands lacked a sufficient bite force capable of penetrating the sclerotized exoskeletons of *P. sarcitis kotoensis*, especially for females and smaller males. As we would expect from insects mimicking *Pachyrhynchus*, the hardness of the longhorn beetle *Doliops similis* from Orchid Island was only 11.5 N (Table S1), which was much lower than that of 'hard' *P. sarcitis kotoensis*.
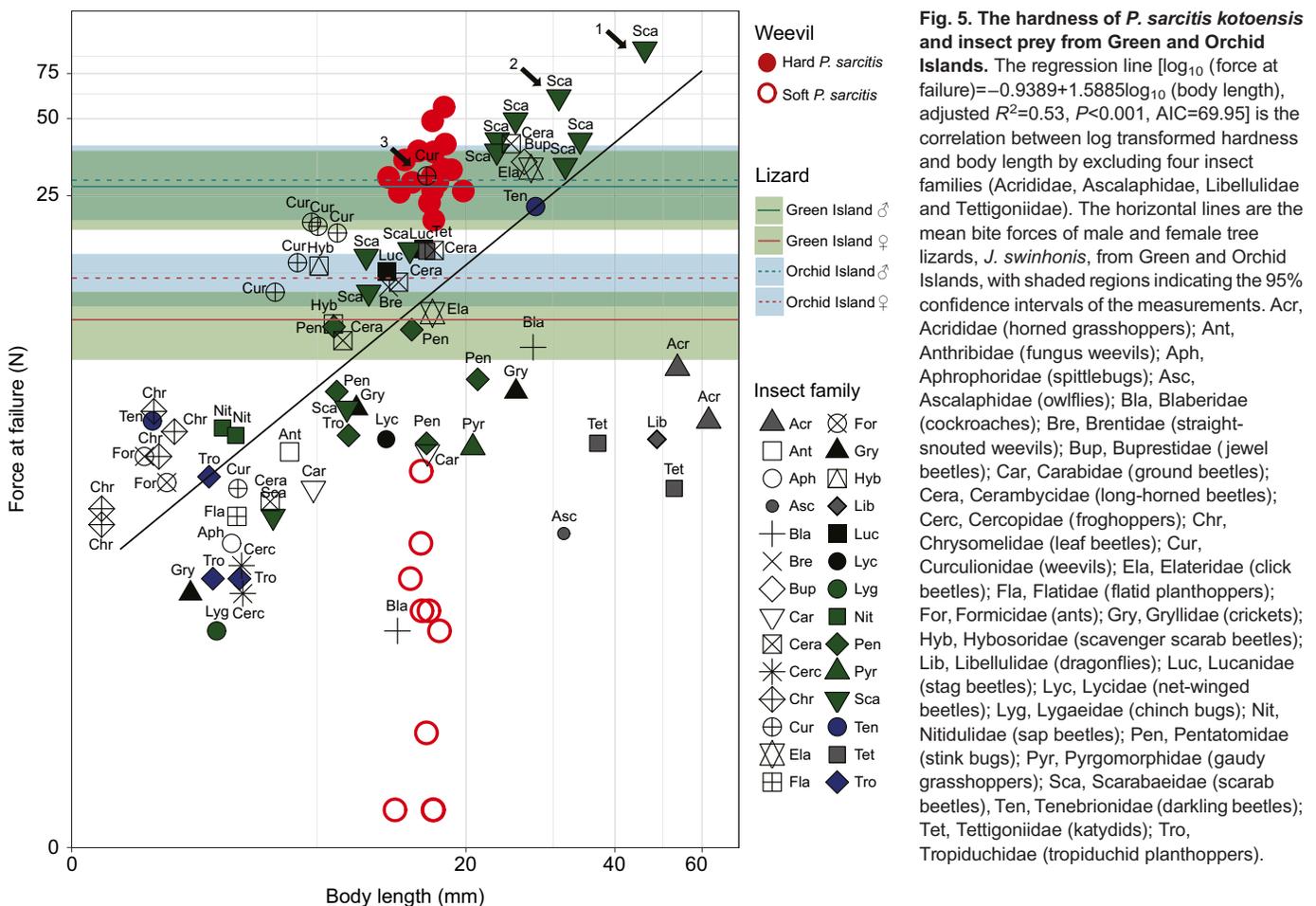
Biochemical compounds of *P. sarcitis kotoensis*

We selected twenty peaks with the highest relative abundance from the master chromatogram of the extraction of *P. sarcitis kotoensis* to compare with the spectrum library (Fig. 6). Five of them, including the solvent [ethane; retention time (RT): 1.72, similarity index (SI): 935, relative similarity index (RSI): 936, Chemical Abstract Service (CAS) number: 60-29-7], had both SI and RSI values higher than 800. The four candidate compounds in *P. sarcitis kotoensis* were identified as ethyl propyl ether, diethylene glycol, butylated hydroxy toluene and cadalene. These chemicals can be found in other animals or plants but have no toxic or repellent functions, and they do not fall into the 13 categories of known insect toxins and repellents against vertebrate predators (Blum, 2012).

DISCUSSION
Effective secondary defence

The study provides the first empirical evidence to support the morphological defence hypothesis pioneered by A. R. Wallace almost 150 years ago. Our findings clearly indicate that the hardness of aposematic *Pachyrhynchus* functions as a secondary defence against predator lizards, whereas the tarsal claw serves no defence function, and no toxins or repellents are stored in the weevil. This secondary defence of hardness is highly effective in at least three intriguing features. First, the hardness of the exoskeleton significantly increased the survival of the weevils under lizard attack. In fact, the survival rate was 100% for the 'hard' weevils. Almost all weevils rejected by lizards were undamaged and resumed walking immediately after the attack. In contrast, the 'soft' weevils were all consumed by the lizards during the staged encounters. These results suggest that the hardness of the exoskeleton is an important fitness component indicative of the morphological adaptation of aposematism in *Pachyrhynchus*. Given that physical attacks by predators are often costly to prey in terms of injury, escaping time or survival (Ruxton et al., 2004), it was very surprising to observe that *Pachyrhynchus* suffered almost no costs in terms of injury and survival. The high survival of *Pachyrhynchus* under lizard attack can provide empirical evidence for the evolutionary origin of aposematic

Fig. 5. The hardness of *P. sarcitis kotoensis* and insect prey from Green and Orchid Islands. The regression line [$\log_{10}$ (force at failure)$=-0.9389+1.5885\log_{10}$ (body length), adjusted $R^2=0.53$, $P<0.001$, AIC=69.95] is the correlation between log transformed hardness and body length by excluding four insect families (Acrididae, Ascalaphidae, Libellulidae and Tettigoniidae). The horizontal lines are the mean bite forces of male and female tree lizards, *J. swinhonis*, from Green and Orchid Islands, with shaded regions indicating the 95% confidence intervals of the measurements. Acr, Acrididae (horned grasshoppers); Ant, Anthribidae (fungus weevils); Aph, Aphrophoridae (spittlebugs); Asc, Ascalaphidae (owlflies); Bla, Blaberidae (cockroaches); Bre, Brentidae (straight-snouted weevils); Bup, Buprestidae (jewel beetles); Car, Carabidae (ground beetles); Cera, Cerambycidae (long-horned beetles); Cerc, Cercopidae (froghoppers); Chr, Chrysomelidae (leaf beetles); Cur, Curculionidae (weevils); Ela, Elateridae (click beetles); Fla, Flatidae (flatid planthoppers); For, Formicidae (ants); Gry, Gryllidae (crickets); Hyb, Hybosoridae (scavenger scarab beetles); Lib, Libellulidae (dragonflies); Luc, Lucanidae (stag beetles); Lyc, Lycidae (net-winged beetles); Lyg, Lygaeidae (chinch bugs); Nit, Nitidulidae (sap beetles); Pen, Pentatomidae (stink bugs); Pyr, Pyrgomorphidae (gaudy grasshoppers); Sca, Scarabaeidae (scarab beetles), Ten, Tenebrionidae (darkling beetles); Tet, Tettigoniidae (katydids); Tro, Tropiduchidae (tropiduchid planthoppers).

colours. One of the puzzles of evolving aposematic colours is that a novel conspicuous individual will be detected easily in the population, and its rarity will make it challenging for predators to learn to avoid the colour signal in future encounters (Fisher, 1958). However, at the beginning of the evolution of warning signals, if a rare, novel conspicuous prey, such as in the case of *Pachyrhynchus*, can survive the initial attack, then the aposematic individual that survived will make it easier for predators to recognize the colour signal; thus, the frequency of warning colour will increase in the prey population (Ruxton et al., 2004).

Second, the hardness of the weevils' exoskeleton immediately triggered strong aversive responses in the lizards after they bit the 'hard' weevils (such as instantly shaking their heads and spitting out the prey). Similar to some of the colourful *Eupholus* and *Trigonopterus* species (Seago et al., 2009; Riedel et al., 2013), the *Pachyrhynchus* species are unique among those highly sclerotized flightless weevils and possess the most diverse aposematic colours (Wallace, 1895; Schultze, 1923). Our findings indicate that this anti-predation strategy is very effective in strengthening the aposematism in colourful *Pachyrhynchus* weevils, by developing a robust association between warning displays and prey unprofitability. Our earlier study found that after only a single encounter, the naïve lizard predators rapidly learned to avoid weevils for at least the next 23 days (Tseng et al., 2014). The lizards can instantly learn from the weevils' unprofitability and reduce future attacks when they encounter prey items with similar warning signals. Therefore, the inclusive fitness of aposematic weevils that confers such defence may subsequently be increased.

Finally, the secondary defence of using the hardness is a 'secret weapon' (*sensu lato*, Eisner et al., 2005) in the sense that, similar to internally stored chemical defences in toxic prey, it is invisible and unexpected by naïve predators. Aposematic prey species that use internally stored toxic chemicals as a source of defence are well known in nature. However, other invisible defence strategies, such as the heavy armature of a harvestman (da Silva Souza and Willemart, 2011) and the hard body of aposematic weevils in this study, are poorly studied (Caro, 2017). Our results suggest that, in addition to surviving the attack, the 'secret weapon' of weevils simultaneously strengthens the lizards' 'unexpectancy' via their outstanding hardness compared with most insect prey with similar and even larger sizes in the same habitat. This high level of hardness largely explains the instant and strong aversive responses displayed by the lizards after biting weevils with their teeth.

Multiple secondary defences

This study showed that the hardness of *Pachyrhynchus* weevils is the main secondary defence against lizard predators. However, like other flightless weevils such as *Trigonopterus* (Van De Kamp et al., 2014), *Pachyrhynchus* employs a series of secondary defensive strategies when encountering human predators; first, it hides itself in the tree branches or leaves, then it performs a sudden drop-off from the trees, and finally, it performs death-feigning on the ground (L.-Y.W. and C.-P.L., personal observations). This series of secondary defensive strategies may be essential in evading predators at the initial stage of prey recognition and pursuit, especially when weevils are soft (i.e. newly emerged without a 'hard' defence) or are encountering
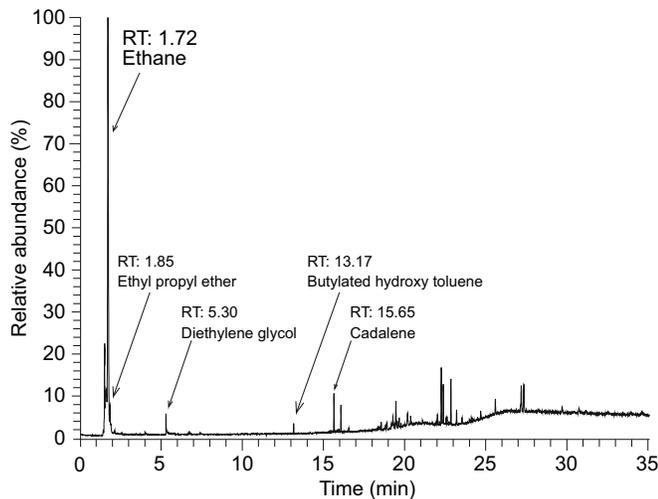
Fig. 6. The master chromatogram and identified candidate compounds from the extraction of *P. sarcitis kotoensis*. The compounds that have SI (similarity index) and RSI (relative similarity index) higher than 800 are shown with retention time (RT). The chemical profile of these compounds are ethane (solvent), RT: 1.72, SI: 935, RSI: 936, CAS registry number: 60-29-7; ethyl propyl ether, RT: 1.85, SI: 867, RSI: 890, CAS number: 628-32-0; diethylene glycol, RT: 5.30, SI: 967, RSI: 967, CAS number: 111-46-6; butylated hydroxy toluene, RT: 13.17, SI: 941, RSI: 945, CAS number: 128-37-0; cadalene, RT: 15.65, SI: 947, RSI: 956, CAS number: 483-78-3.

predators with greater bite forces. Nevertheless, it is still unknown whether 'soft' weevils could perform drop-off and death-feigning equally well as 'hard' individuals.

Mechanical mechanism of hardness

The mechanical mechanism of achieving the outstanding hardness for a small-sized insect such as *Pachyrhynchus* remains elusive. The mechanical properties of the insect's cuticle, such as strength and stiffness, depend on many physical and chemical factors, including the arrangement of cuticle microfibres, protein and water content, and process of pigmentation and sclerotization (Andersen, 2010; Klocke and Schmitz, 2011). A recent study indicated that an endosymbiont *Nardonella* specialized in the cuticle formation and hardening of *Pachyrhynchus* hosts by providing tyrosine, suggesting the important role of bacterial symbiosis in the weevil's hardness (Anbutsu et al., 2017). The microstructure of the elytral cuticle in weevils is phylogenetically conserved to have a 'weevil-specific' combination of interlocking of exocuticles and endocuticles, an endocuticle with distinct ovoid macrofibres embedded in a matrix, and small angles between successive endocuticular layers (Van de Kamp et al., 2016). These microstructural attributes, or their modifications, may have contributed to the hardness of *Pachyrhynchus*. However, the cuticular microstructures in other hard flightless weevils, such as *Trigonopterus*, do not deviate from the weevil's uniform ground plan but instead possess thicker elytra (Van de Kamp et al., 2016). In addition to cuticular microstructures and thickness, sclerotization (Hopkins and Kramer, 1992), heavy metals and halogens (Schofield et al., 2002) can enhance the mechanical strength of insect cuticles. The mechanical behaviour of an insect's body is the result of combining material properties and geometric characteristics. Thus, the geometry of an organism can modify the distribution of stress and reduce the concentration of stress to prevent mechanical failure (e.g. coiling mollusc shells in Rajabi et al., 2014). Because the *Pachyrhynchus* species have a peculiar dome-shaped thorax and

abdomen and densely interlocked elytra, these oval architectures may equip these weevils with more robust bodies as a defence strategy against predators.

Adaptive defence against lizard predation

This study provided convincing evidence indicating that the hardness of aposematic *Pachyrhynchus* weevils is ecologically important and represents an adaptive defence mechanism against predation by tree lizards. The results suggested that the hardness of the weevils was among the highest in the prey spectrum of the tree lizards and higher than the average bite force of the lizards from the Green and Orchid Islands. Therefore, predation by small vertebrate predators, such as tree lizards, may have acted as a primary driving/maintaining force for the evolution of hardness in *Pachyrhynchus* weevils. At present, *Japalura swinhonis* is the only known natural predator of *Pachyrhynchus* weevils on these two islands (Huang, 2007). The other eight lizard species on the islands are less likely to be major predators of the weevils due to their habitat and prey preference (Chen et al., 2008; Li et al., 2010) (Table S2). The hard body of *Pachyrhynchus* probably originated in the common ancestors of relatives, such as *Eupyrgops*, *Metapocyrtus* and *Polycatus* in the Philippine Archipelago and neighbouring islands (Schultze, 1923); therefore, the arboreal lizards of Southeast Asia, such as *Gekko* species, might also be important in promoting the origin of the weevils' hard exoskeletons.

Multiple selective forces of the diverse predator community may each play an important role in driving and maintaining the evolution of aposematic colours and the associated defence strategies (Willink et al., 2014). In addition to lizard (reptilian) predation, avian and mammalian predation constitute the other two major selective forces on tropical insects with aposematic or cryptic colouration (Mappes et al., 2014; Roslin et al., 2017). Avian predation is likely to be essential for the evolution of aposematism and defence strategies in *Pachyrhynchus* weevils because, in general, birds possess a wide range of colour spectra (Bennett and Théry, 2007), and they are primary predators of aposematic insects (Iniesta et al., 2017). In the Green and Orchid Islands, there are approximately 62 bird species that eat insects as part of their diets (Chen et al., 2008; Li et al., 2010; Severinghaus et al., 2012) (Table S3). Of these, 12 species [emerald dove (*Chalcophaps indica*), large hawk-cuckoo (*Cuculus sparverioides*), Himalayan cuckoo (*Cuculus saturatus*), lesser coucal (*Centropus bengalensis*), common kingfisher (*Alcedo atthis*), brown shrike (*Lanius cristatus*), Japanese paradise-flycatcher (*Terpsiphone atrocaudata*), brown-eared bulbul (*Microscelis amaurotis*), lowland white-eye (*Zosterops meyeni*), white-shouldered starling (*Sturnus sinensis*), eyebrowed thrush (*Turdus obscurus*), and blue rock thrush (*Monticola solitarius*)] inhabit the same habitat and are probable avian predators of *Pachyrhynchus* (Table S3). At present, no bite forces of avian fauna from Green and Orchid Islands are available for comparison. However, the bite forces of the vertebrates are often strongly correlated with head sizes and shapes (Anderson et al., 2008). The average bite force of small- to median-sized passerines is about 9.3 ±10.5 N (2.9–38.4 N, *N*=18, Estrildidae and Fringillidae; Van der Meij and Bout, 2006), suggesting that the hardness of *Pachyrhynchus* weevils (32.6±9.1 N) may be effective against predation by most passerine birds of similar sizes. Nevertheless, the shape of beaks and pecking behaviour of most birds alone may be effective to penetrate the exoskeleton of *Pachyrhynchus*. Our field observations suggested that *Pachyrhynchus* weevils were defenceless and could be easily pecked and swallowed by adult domestic chickens. However, smaller domestic chicks were

observed to spend a considerable amount of time pecking the weevils, but they could not successfully swallow them. These preliminary observations together with the bite force measurement of birds suggest that the hardness of *Pachyrhynchus* weevils may only be effective against smaller avian predators in their habitats.

There are 14 mammal species found in the Green and Orchid Islands that may consume insects (Chen et al., 2008; Li et al., 2010), of which 10 species [masked palm civet (*Paguma larvata*), Chinese white-toothed shrew (*Crocidura rapax tadae* and *C. r. lutaoensis*), Pallas's squirrel (*Callosciurus erythraeus taiwanensis*), house mouse (*Mus musculus*), lesser rice field rat (*Rattus losea*), Tanezumi rat (*R. tanezumi*), greater bandicoot rat (*Bandicota indica*), brown rat (*R. norvegicus*) and black rat (*R. rattus*)] occur in the same habitat as *Pachyrhynchus*. Tanezumi rats were observed to forage on tree trunks in the weevil's habitat during the day and night-time, and they are likely to be one of their mammalian predators, with bite forces capable of penetrating their exoskeletons (24.7–76.8 N, brown rat, *R. norvegicus*; Cox et al., 2012). Future studies on identifying the major predators and assessing spatio-temporal variation in predation in *Pachyrhynchus* weevils will help us to better understand the ecology and evolution of the hardness in aposematic prey.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: W.-S.H., C.-P.L.; Methodology: W.-S.H., H.-C.T., L.-C.H., C.-P.L.; Validation: L.-Y.W., C.-P.L.; Formal analysis: L.-Y.W.; Investigation: L.-Y.W., W.-S.H., H.-C.T., L.-C.H., C.-P.L.; Resources: W.-S.H., H.-C.T., L.-C.H., C.-P.L.; Data curation: L.-Y.W., H.-C.T., L.-C.H.; Writing - original draft: L.-Y.W.; Writing - review & editing: C.-P.L.; Visualization: L.-Y.W.; Supervision: W.-S.H., C.-P.L.; Project administration: C.-P.L.; Funding acquisition: W.-S.H., C.-P.L.

Data availability
Data from this study are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.73kq1.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.172486.supplemental

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