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LETTER

Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack

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Abstract

The roots of most land plants are colonised by mycorrhizal fungi that provide mineral nutrients in exchange for carbon. Here, we show that mycorrhizal mycelia can also act as a conduit for signalling between plants, acting as an early warning system for herbivore attack. Insect herbivory causes systemic changes in the production of plant volatiles, particularly methyl salicylate, making bean plants, *Vicia faba*, repellent to aphids but attractive to aphid enemies such as parasitoids. We demonstrate that these effects can also occur in aphid-free plants but only when they are connected to aphid-infested plants via a common mycorrhizal mycelial network. This underground messaging system allows neighbouring plants to invoke herbivore defences before attack. Our findings demonstrate that common mycorrhizal mycelial networks can determine the outcome of multitrophic interactions by communicating information on herbivore attack between plants, thereby influencing the behaviour of both herbivores and their natural enemies.

Keywords

Arbuscular mycorrhizal fungi, broad bean (*Vicia faba*), common mycelial networks, induced defence, multitrophic interactions, parasitoid wasp (*Aphidius ervi*), pea aphid (*Acyrthosiphon pisum*), plant volatiles, plant-to-plant communication.

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INTRODUCTION

Arbuscular mycorrhizal (AM) fungi form symbioses with many herbaceous plants, including important crop species, have a near global distribution and are among the most functionally important soil microorganisms (Smith & Read 2008). AM fungi often significantly improve mineral nutrient uptake (Smith & Read 2008) and can enhance tolerance to root and shoot pathogens (Whipps 2004), nematodes (De La Peña *et al.* 2006; Vos *et al.* 2012) and drought (Smith & Read 2008). In return, plants supply AM fungi with carbohydrates (Johnson *et al.* 2002) that are used in part to develop extensive mycelial networks (Leake *et al.* 2004), which act as conduits for carbon (Johnson *et al.* 2002) and mineral nutrients (Johnson *et al.* 2001). Due to a lack of specificity of AM fungi to their host plants (Smith & Read 2008), external mycelia can produce so-called 'common mycelial networks' that connect the roots of different species, as well as individuals of the same species (Simard & Durall 2004). Common mycelial networks facilitate seedling establishment (Van Der Heijden 2004), influence plant community composition (Van Der Heijden & Horton 2009) and are the primary pathways through which many species of non-photosynthetic plants acquire their energy (Bidartondo *et al.* 2002).

Evidence is emerging that mycelial networks have potential to transport signalling compounds. Barto *et al.* (2011) demonstrated that allelochemicals released by marigold (*Tagetes tenuifolia* Millsp) could be transported through AM fungal networks to inhibit the growth of neighbouring plants. Song *et al.* (2010) found that interplant connections via common mycelial networks led to increased disease resistance, defensive enzyme activities and defence-related gene expression in healthy tomato plants (*Lycopersicon esculentum* Mill) connected to plants infected with leaf early blight (*Alternaria solani*). This finding suggests that interplant transfer of pathogenic fungal disease resistance signals via these networks could be occurring.

If common mycelial networks can act as conduits for signalling compounds, there clearly is considerable potential for mycorrhizal fungi to mediate plant responses to herbivores. There could also be effects on other trophic levels such as herbivore enemies, because both insect herbivores and their parasitoid enemies respond to volatile organic compounds (VOCs) emitted by plant leaves albeit in different ways. Sap-sucking herbivores such as aphids use VOCs as cues for locating host plants (Bruce *et al.* 2005) but, following the attack, the composition of VOCs released changes and becomes repellent to subsequent herbivores (Bernasconi *et al.* 1998) and attractive to their natural enemies, such as parasitoid wasps (Turlings *et al.* 1995). VOCs produced by infested plants are often produced systemically (Pickett *et al.* 2003) and can be transmitted aerially between plants (Dicke & Bruin 2001) as well as being released into the rhizosphere from roots (Chamberlain *et al.* 2001; Rasmann *et al.* 2005).

It has been proposed (Barto *et al.* 2012; Dicke & Dijkman 2001), but so far not tested, that common mycelial networks may facilitate interplant transfer of signalling compounds released by plants under attack by insect herbivores and that such signalling may induce emission of VOCs. If so, this could potentially have profound effects on multitrophic interactions. Here, we test the hypothesis that common mycelial networks act as interplant conduits that provide an early warning system of herbivore attack. We quantify effects on the behaviour of a piercing, sucking aphid herbivore and

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one of its key natural enemies, and identify the chemical signal driving the insect behavioural responses. Specifically, we test how common mycelial networks linking aphid-infested plants with aphid-free plants affect the attractiveness of VOCs to aphids and parasitoid wasps. If AM fungi act as conduits for signalling compounds between aphid-infested plants and uninfested plants, we predict that aphid-free plants connected via common mycorrhizal networks to aphid-infested plants will act as if they themselves are infested, that is, they will share similar VOC profiles and elicit similar responses from insects. We also expect VOCs that are repellent to aphids to be attractive to parasitoid wasps. This is because these VOCs are produced in response to aphid infestation, acting to repel further aphid attack and to attract natural enemies, since they are a signal that the parasitoid's aphid prey is present.

MATERIALS AND METHODS

Mesocosm establishment

Eight mesocosms (30 cm diameter) were established in a greenhouse containing a mix of 10% loam top soil [all nutrients solely from the base materials: 9% clay, 17% silt, 74% sand, pH = 7.8, organic matter 24.2%, total nitrogen (Dumas) 0.74%, available phosphorus 64 mg L^{-1}, available potassium 1324 mg L^{-1}, available magnesium 222 mg L^{-1}], 24% sand, 16% terra green and 10% grit all from LBS (Colne, UK) and 40% washed sand from Culbin Forest National Nature Reserve, Morayshire, UK, and an inoculum of the AM fungus *Glomus intraradices* UT118 (INVAM). Seedlings of *Plantago lanceolata* L. were used to establish a mycorrhizal fungal network in each mesocosm for 4 months prior to the experiment, after which all their shoots were removed.

Experimental design

Five eleven-day-old seedlings of bean (*Vicia faba* L.) cultivar 'Sutton dwarf' (Moles seeds, Colchester, UK) were planted in the mesocosms (Fig. 1). The beans were arranged so that a central plant acted as a 'donor', which received aphids in the last 4 days of the experiment, surrounded by four 'receiver' plants that never came into direct contact with aphids (Fig. 1). Two receiver plants were controls whose mycorrhizal fungi were not connected to the donor (achieved by two independent methods), and two receivers were connected to the donor by the common mycelial network (also using two methods). In one control treatment, the receiver plant was grown in a core (6 cm diameter, 20 cm deep) surrounded by 0.5 μm mesh, preventing penetration by fungal hyphae such that external mycorrhizal mycelium from the plant could never form connections with neighbours. The second control receiver plant was grown in a core surrounded by 40 μm mesh. This mesh enabled the plants to form common mycelial networks but, immediately before aphids were added to the donor plants, the core was rotated to snap all fungal hyphae penetrating through the mesh (Johnson *et al.* 2001), thus breaking the connection with the donor plant. The two other receiver plants could form common mycelial networks with the donor plant: one grown with no barrier, allowing the intermingling of both mycorrhizal mycelium and roots with the donor, and one allowing mycelial contact only by means of a 40 μm mesh core that was never rotated. This enabled us to separate any potential plant-to-plant signalling via root contact from signalling via common mycelial networks. A key aspect of this design is that all plants were colonised by AM fungi to avoid issues arising from known differences in composition of volatiles between mycorrhizal and non-mycorrhizal plants (Guerrieri *et al.* 2004). Microscopical examination of trypan blue-stained roots confirmed that all bean plants were colonised by AM fungi.

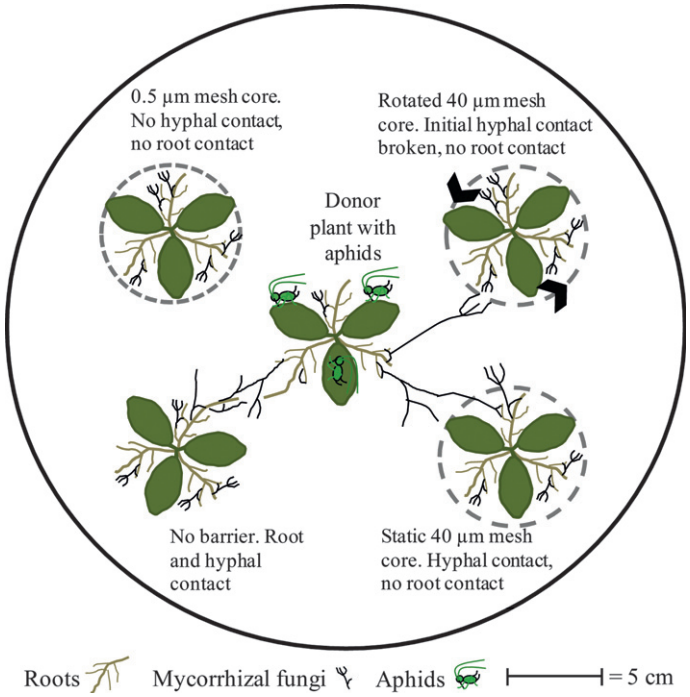

Figure 1 Experimental mesocosm (30 cm diameter; $n = 8$) showing the donor plant, which was colonised by aphids, and four aphid-free receiver plants. All plants were grown in the mycorrhizal condition but one plant was prevented from forming mycelial connections to donor plants (0.5 μm mesh), another was allowed to form connections initially but the connections were snapped after additions of aphids to the donor (rotated 40 μm mesh), and two other plants were allowed to form shared mycorrhizal fungal networks (non-rotated 40 μm mesh allowing fungal contact only; no barrier allowing fungal and root contact) with the donor plant for the duration of the experiment.

Five weeks after transplanting the *V. faba* plants, by which time hyphal connections would have been well established, all receiver plants were placed in polyethyleneterephthalate (PET) bags, which prevented plant-to-plant communication via aerial volatiles, and connected to entrainment apparatus (see section on collection of volatiles below) immediately before the donor plant was infested with 50 adult pea aphids (*Acyrthosiphon pisum* Harris), before itself being sealed with a bag. The aphids were supplied by Rothamsted Research Institute and were reared on broad beans in the laboratory at the University of Aberdeen (20 ± 3 °C; 16 h day: 8 h dark).

Collection of plant volatiles

Collection of volatiles (Bruce *et al.* 2008) was conducted on all plants 96 h after addition of aphids on the donor plant using an air entrainment kit (BJ Pye, Kings Walden, UK). This timing is based on findings that expression of plant defence genes occurs 2–3 days after aphid attack (e.g. De Vos *et al.* 2005). Plant shoots were enclosed in PET bags, heated to 180 °C for at least 2 h before use, which were fastened around the stems using polytetrafluoroethylene (PTFE)

tape. Air, purified by passage through an activated charcoal filter, was pumped into the bag through an inlet port made of PTFE tubing at 600 mL min^{-1}. Volatiles were collected using pre-conditioned (dichloromethane) glass tubes containing Porapak Q polymer (50 mg) inserted into collection ports fitted in the top of the bag. Air was pumped through these tubes at 400 mL min^{-1}, less than the input rate, thus ensuring that unfiltered air was not drawn into the collection bag from outside, but which will have resulted in a capture efficiency of about 66%. The bags and pumped air served to prevent aerial volatiles causing communication between plants, so that our observations were the result of below-ground rather than above-ground conduits. The period of entrainment collection was 24 h. Porapaq Q filters were eluted with 0.5 mL of diethyl ether (spectrophotometric grade, inhibitor free, Sigma Ltd) and stored at −20 °C. Five background headspace samples were obtained using an identical procedure but without any plants. Headspace sampling allowed isolation of the volatiles from plants exposed to different treatments to enable accurate assessment of insect responses to those volatiles in subsequent bioassays. Doses used in bioassays were adjusted so that they were ecologically relevant in terms of the amount in plant equivalents over the duration of the bioassay.

Behavioural responses of aphids and parasitoids

Parasitoids were reared on pea aphids on broad beans in the insectary at Rothamsted Research (22 ± 3 °C; 16 h day: 8 h dark). To test whether aphids and parasitoids were attracted or repelled by headspace samples collected from donor and receiver plants, we conducted bioassays using a four-arm olfactometer (Pettersson 1970; Webster *et al.* 2010) either using alate (winged) morphs of pea aphids starved for 2–4 h prior to the bioassay, or female parasitoid wasps (*Aphidius ervi* Haliday), which had experience of oviposition. Filters paper treated with reagent blanks were attached to three of the arms, while paper treated with 10 μL of VOCs eluted from plant headspace gas samples was attached to the remaining arm. The insect was placed inside the central area and air was pulled through the apparatus by a suction pump (200 mL min^{-1}). Insect movement in the arena was recorded using OLFA (Exeter Software, Setauket, NY, USA) software during the bioassay. Each bioassay was conducted for 16 min, and each 2 min, the olfactometer was turned 45° in one direction to avoid any bias caused by uneven light. The 10 μL VOC sample was 1/35th of the volume collected per 24 h entrainment, and together with the capture efficiency, we estimate the dose in the bioassay was 1.7 times the amount produced by the plants under the experimental conditions. The attractiveness of plant headspace samples to insects was taken as the time spent by the insect in the olfactometer area containing plant headspace samples, minus the time spent in the olfactometer area treated with reagent blanks.

For pea aphids, samples from seven of the eight replicates of each treatment were used, and we performed five bioassays per sample (for statistical analyses, means of these five bioassays were used). For parasitoids, we used VOC samples from between six and eight replicates per treatment, and performed between three and five bioassays per sample. We always used a fresh preparation of VOCs sample on the filter paper and a new insect for each bioassay. This was the highest possible replication of bioassays allowed by the volumes of VOCs samples we collected. We conducted further behavioural bioassays with aphids only, due to limited amounts of remaining headspace samples, to test the effect of authentic standards of the identified chemicals in driving insect responses. Analysis of VOCs eluted from plant headspace samples indicated that methyl salicylate may play a role in insect response to plants. To test this potential chemical mechanism, we added 3 ng mL^{-1} of methyl salicylate (the mean concentration found in samples that were naturally repellent to aphids) to head-space samples that were originally attractive to aphids, and undertook additional bioassays following the protocol described previously.

Gas chromatography (GC) analysis of plant headspace VOCs

Separation of VOCs from each plant headspace sample was achieved on a non-polar (HP-1, 50 m × 0.32 mm inner diameter × 0.5 mm film thickness, J & W Scientific) capillary column using an HP6890N GC (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated retention gap (1 m × 0.53 mm inner diameter) and flame ionisation detector (FID). The carrier gas was hydrogen. Samples (2 μL) were injected using an HP 7683 series injector. The amounts of VOCs produced per plant were quantified using external standards.

Identification of electrophysiologically active VOCs

Electroantennography (EAG) recordings from aphid and parasitoid antennae ($n \geq 3$ preparations) coupled to a gas chromatograph (GC-EAG; Wadhams 1990; Sasso *et al.* 2009) were used to identify active VOCs eliciting a response from the insects. EAG recordings were made using Ag-AgCl glass electrodes filled with a saline solution (as in Maddrell 1969, but without glucose). For both aphids and parasitoids, the head was excised and placed within the indifferent electrode, and the tips of both antennae were removed before they were inserted into the recording electrode. The effluent from the transfer line to the antenna was delivered into a purified airstream (1.0 L min^{-1}) flowing continuously over the preparation. Separation of the volatiles from each plant headspace sample was achieved on an AI 93 GC equipped with a cold on-column injector and FID. The carrier gas was helium and the VOCs were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands) and analysed using the software package Syntech. Compounds were assumed to be EAG-active if they caused EAG responses on three or more preparations.

GC coupled mass spectrometry (GC-MS) analysis of electrophysiologically active VOCs

GC-EAG recordings were used to determine which peaks of the GC separation elicited electrophysiological responses from aphid and parasitoid antennae, and identification of the active peaks was achieved by GC on a capillary column (50 m × 0.32 mm i.d., HP-1) directly coupled to a mass spectrometer (GC-MS; AutospecUltima, Micromass, UK). Tentative GC-MS identifications were confirmed by peak enhancement with authentic standards on two GC columns of differing polarity. The stereochemistry of linalool and germacrene D was determined using an HP 5890 GC equipped with a cool-on-column injector and FID, fitted with a β-cyclodextrin chiral capillary column (Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness). After confirming that successful separation of synthetic enantiomers was accomplished, co-injections were carried out. Peak enhancement confirmed the presence of the enantiomer in the headspace sample. The

identity of EAG-active compounds was confirmed by repeating the EAG analysis using a synthetic blend of the VOCs (see Appendix S1 in Supporting Information). Chemicals used for peak enhancements were from the same sources as the chemicals used for the synthetic blend (Appendix S1). There were no EAG-active VOCs in the plant-free background control samples.

Statistical analysis

The behavioural response of insects was tested in two ways. First, the attractiveness or repulsion of each EAG-active VOC from headspace samples was tested by paired t-test. In this analysis, we compared the time spent by each insect in olfactometer compartments containing VOCs from headspaces compared to compartments containing reagent blanks. The second approach tested for differences between treatments in the attractiveness to insects. Here, the time spent by insects in olfactometer compartments containing VOCs from headspace samples was subtracted from the time spent in compartments containing reagent blanks, and the resulting data (means per plant) were analysed using a general linear model (GLM) with treatment (i.e. corresponding to the five different plants within a mesocosm) as a fixed factor and mesocosm as a random factor. Least significant difference (LSD) *post hoc* tests were applied to examine pair-wise differences in the attractiveness of plant headspace samples between treatments. We also explored the relationship between aphid and parasitoid responses using linear regression. All analyses were run in PASW (SPSS) 19 package.

The composition of VOCs produced by the plants was analysed by principal component analysis (PCA) on a correlation matrix consisting of log transformed amounts (g dwt^{-1}) of EAG-active VOCs, obtained from all replicates, using the prcomp function in R version 2.3.7.1 (R Development Core Team 2008). This distilled the 17 EAG-active VOCs into a smaller number of groupings, or principal components (PCs). We used two types of output from the PCA: the first was a matrix of 'loadings' for each of the 17 EAG-active VOCs obtained for each of the PCs. These loadings aided biological interpretation of the PCA, because they indicate the strength of correlation between individual VOCs and each PC. The second was a matrix of 'scores', with a single score representing each replicate headspace VOC sample for each PC. To explore potential chemical mechanisms driving the insect behavioural response to treatments, scores from each of the first five PCs associated with each plant's headspace EAG-active VOCs were tested against behavioural responses of both aphids and parasitoids using linear regression. We also used a GLM (using the lm function in R) to test for effects of treatment on the scores of the first five PCs. In this analysis, there were three treatment groups comprising unconnected plants (i.e. plants in 0.5 μm mesh and rotated 40 μm mesh cores), connected plants (i.e. plants in static 40 μm mesh cores and in bulk soil) and donor plants. Pair-wise comparisons were achieved by re-levelling of the order of treatments in the analysis. As a further test of the effect of treatment on plant-emitted VOCs (normalised g dwt^{-1}), we tested for differences in the amounts of individual EAG-active VOCs between donor, connected and unconnected plants using a nonparametric Kruskal–Wallis test in SPSS, because the data did not meet assumptions for homogeneity of variances for parametric tests.

Differential responses of pea aphids to attractive samples before and after addition of methyl salicylate were tested using GLM with methyl salicylate addition (i.e. with or without addition) as a fixed factor and plant as a random factor (SPSS). Assumptions for using GLMs were validated by plotting residuals vs. fitted values, square root residuals vs. fitted values, normal qq plot and constant leverage.

RESULTS

Behavioural responses of aphids and parasitoids

Headspace samples collected from aphid-infested donor plants were significantly repellent to aphids ($P < 0.001$; $t_{1,34} = -5.73$) and attractive to parasitoids ($P < 0.001$; $t_{1,26} = 4.49$; Fig. 2a).

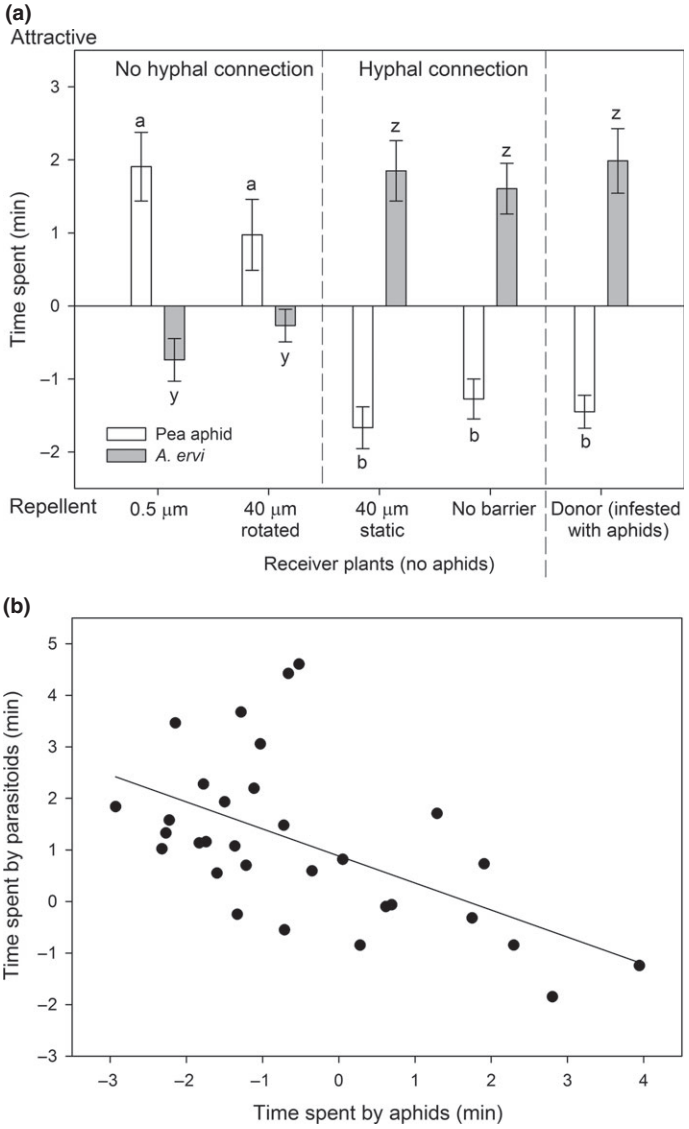

Figure 2 Behavioural responses of pea aphid and the parasitoid wasp *A. ervi* to volatile organic compounds from the headspace of experimental plants. (a) Mean time spent in olfactometer compartments containing volatiles from treated plants minus reagent blanks (± SE). Pea aphid and *A. ervi* responses are compared separately. Bars sharing a letter are not significantly different from each other ($P > 0.05$); (b) Relationship between mean time spent in olfactometer arms by pea aphids and *A. ervi* across all treatments (Pearson coefficient = −0.553; $P = 0.001$).

Crucially, when aphid-free receiver plants were connected to donor plants by common mycelial networks, headspace samples collected from the connected receivers were significantly repellent to aphids, regardless of whether the plants were connected by external mycelium only (non-rotated 40 μm mesh cores; $P < 0.001$; $t_{1,34} = -6.04$) or by root contact and external mycelium (i.e. plants in bulk soil; $P < 0.001$; $t_{1,34} = -4.32$). Parasitoid wasps gave the opposite response and were significantly attracted to VOCs from plants in the non-rotated 40 μm mesh cores ($P < 0.001$; $t_{1,28} = 4.48$) and bulk soil ($P < 0.001$; $t_{1,27} = 4.62$; Fig. 2a). Thus, insect behavioural responses to aphid-free receiver plants with a hyphal connection were similar to those for the aphid-infested plants themselves. In contrast, headspace samples from control receiver plants that had no hyphal connection to donor plants were significantly ($P < 0.001$, $F_{4,24} = 21.1$) attractive to aphids compared to infested donors or to receivers that were connected to infested donors with hyphae (Fig. 2a). This effect occurred regardless of the method used to prevent formation of fungal networks.

The responses of parasitoids were also significantly affected by treatment ($P < 0.001$, $F_{4,24} = 6.67$) and were opposite to the responses of aphids, as often occurs with insect responses to herbivore-induced volatiles. We also found a significant negative correlation between aphids and parasitoids in how attractive they found each sample of volatiles (Pearson coefficient = −0.553; $P = 0.001$; Fig. 2b). In addition, we found that headspace samples from receiver plants that had contact with donors via common mycorrhizal fungal networks only (plants in the non-rotated 40 μm mesh cores) were as repellent to aphids as were headspace samples from plants where roots could also intermingle between donors and receivers ($P = 0.461$; Fig. 2a), implying that direct root contact and soil diffusion were not significant additional conduits of signalling.

Effect of treatments on VOCs and their association with behavioural responses of aphids and parasitoids

We identified 17 VOCs that were EAG-active with aphid and parasitoid antennae (Table 1). The first five PCs from the PCA analysis of VOCs accounted for 79% of variance in the data. There was no effect of treatment on PC1, PC2, PC4 and PC5 scores. However, there was a significant effect of treatment on PC3 scores ($F_{2,39} = 5.15$; $P = 0.011$), which accounted for 12% of variance in the data, with higher scores in connected receiver plants ($P = 0.008$) and donor plants ($P = 0.013$) than unconnected receiver plants; there was no difference in PC3 scores between connected plants and donor plants ($P = 0.747$; Fig. 3). PC3 was not only negatively correlated to aphid behavioural responses ($P = 0.046$, $F_{1,34} = 4.3$; Fig. 4a) but also positively correlated with parasitoid behavioural responses ($P = 0.004$, $F_{1,34} = 9.4$; Fig. 4b). The greatest loadings of PC3 were for methyl salicylate (0.42), naphthalene (−0.40), (*E*)-β-farnesene (0.38) and 6-methyl-5-hepten-2-one (0.35), which indicates that these were the main VOCs contributing to PC3 and to insect behavioural responses. Methyl salicylate was found to be the only compound that was EAG active with both aphids and parasitoids, and had concentrations in plant headspace samples that differed significantly ($P = 0.015$) between connected and unconnected receiver plants (Table 1). Quantitative differences in production of all other VOCs between treatments were not significant. Methyl salicylate, which had greatest loadings with PC3, was also a constituent of VOCs from donor plants and plants connected with common mycelial networks. Addition of methyl salicylate to samples originally attractive to aphids (using the mean quantity that occurred in the repellent samples) made them become repellent to aphids ($F_{1,7} = 118.4$; $P < 0.001$; Fig. 5). This chemical

Table 1 The mean amounts (ng g^{-1} dwt per 24 h ± SEM) of volatile organic compounds collected from the headspace of plant shoots in response to treatments, and electrophysiological activity with aphids and parasitoids

				No hyphal connection		Hyphal connection		
Volatile compounds	Kovats index	Aphid	Parasitoid	0.5 μm	40 μm rotated	40 μm static	No barrier	Aphid-infested donor
(*Z*)-2-hexenal	817	+	-	2.16 ± 0.65	3.02 ± 0.50	3.07 ± 0.23	3.67 ± 0.67	3.26 ± 0.56
(*E*)-2-hexenal	825	+	+	0.47 ± 0.20	1.26 ± 0.41	1.28 ± 0.30	1.18 ± 0.54	0.80 ± 0.33
(*E,E*)-2,4-hexadienal	880	+	-	0.10 ± 0.06	0.25 ± 0.12	0.22 ± 0.07	0.59 ± 0.27	0.46 ± 0.23
(*Z*)-2-heptenal	924	+	-	2.59 ± 1.48	0.93 ± 0.53	0.50 ± 0.27	0.90 ± 0.68	2.00 ± 0.96
benzaldehyde	929	+	+	44.9 ± 22.3	17.4 ± 8.5	76 ± 57.23	117.7 ± 86.8	7.69 ± 2.58
6-methyl-5-hepten-2-one	967	+	#	1.84 ± 0.93	2.46 ± 1.23	1.49 ± 0.88	5.92 ± 2.23	7.26 ± 3.73
(*R,S*)-β-pinene	972	+	-	8.22 ± 5.33	10.49 ± 5.86	6.1 ± 5.15	11.12 ± 5.64	1.47 ± 0.39
(*Z*)-3-hexenyl acetate	986	+	+	34.4 ± 18.2	14.7 ± 9.5	7.7 ± 3.1	22.5 ± 15.7	19.6 ± 11.4
3-carene	1009	-	+	9.26 ± 1.19	8.88 ± 0.85	9.04 ± 0.38	8.52 ± 1.09	8.00 ± 1.18
(*S*)-linalool	1086	+	+	40.8 ± 27.5	15.3 ± 12.2	0.63 ± 0.31	15.1 ± 13.1	4.41 ± 2.86
naphthalene	1168	+	+	11.1 ± 6	4.97 ± 1.83	1.66 ± 0.81	5.60 ± 2.72	2.66 ± 1.33
methyl salicylate*	1172	+	+	0.06 ± 0.06	0.41 ± 0.25	1.85 ± 1.10	1.42 ± 0.90	1.46 ± 1.04
cinnamaldehyde	1232	+	+	27.5 ± 15.3	19.7 ± 10.6	3.72 ± 1.79	5.96 ± 2.99	35.3 ± 18.6
(*E*)-caryophyllene	1424	+	-	210.5 ± 96.5	86.6 ± 47.7	107 ± 92.2	155.5 ± 54.6	57.7 ± 21.3
(*E*)-β-farnesene	1450	+	#	1.38 ± 0.45	0.77 ± 0.27	1.84 ± 1.08	2.75 ± 0.84	3.06 ± 1.07
(*R*)-germacrene D	1486	+	-	63.6 ± 41	29.8 ± 19.3	39.9 ± 38.1	31.3 ± 15.7	9.4 ± 7.5
(*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	1570	+	+	466 ± 81.3	185 ± 39	109.1 ± 28	451.7 ± 164.8	416.9 ± 140.1

+Indicates volatiles electrophysiologically active with either pea aphids or parasitoids in these experiments.
#Indicates compounds that are electrophysiologically active with parasitoids according to published data (Du *et al.* 1998).
-Indicates compounds that showed no electrophysiological activity.
*Significant difference ($P = 0.01$; $H = 6.5$) between receiver plants connected and unconnected to the donor (Kruskal–Wallis test).

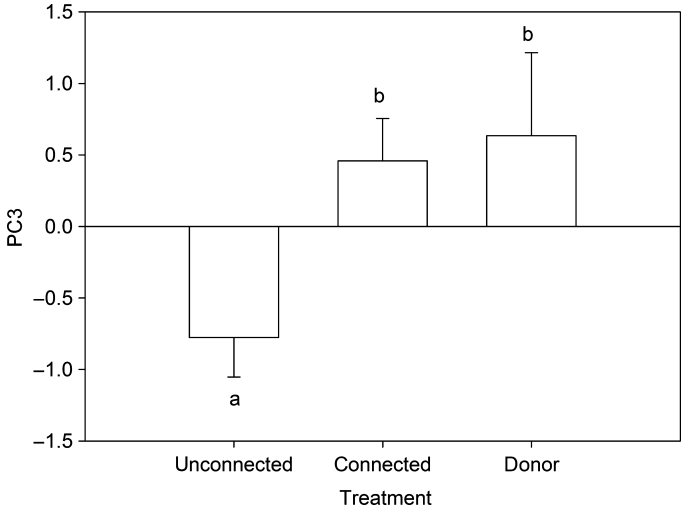

Figure 3 Mean (+SE) principal component 3 (PC3) scores derived from the amounts of electrophysiologically active volatiles produced in donor plants, plants connected to donors (plants in static 40 μm mesh cores and in bulk soil) and plants unconnected to donors (plants in 0.5 μm mesh and rotated 40 μm mesh cores). Bars sharing a letter are not significantly different ($P > 0.05$).

manipulation provides strong support that methyl salicylate is a key aerial compound driving aphid behaviour.

DISCUSSION

We present the first experimental evidence that herbivore-induced signalling molecules can be transferred from plants infested with aphids to uninfested neighbours via a common mycelial network. Our data show that presence or absence of hyphal connections play a vital role in determining the response of receiver plants connected to aphid-infested donors. The use of a mycotrophic plant species, a vigorous AM fungal inoculum for colonisation of roots, and an initially sterile substrate maximises the likelihood that mycorrhizal rather than non-mycorrhizal fungi were the key agents in the transfer of signal molecules between plants. While non-mycorrhizal fungi might have colonised plant roots and contributed to the transfer of signalling compounds, this is unlikely because AM fungi often antagonise soil pathogenic fungi (e.g. Bharadwaj *et al.* 2012; Campos-Soriano *et al.* 2012; Jung *et al.* 2012) and the bean roots were confirmed to contain abundant arbuscules, which are specific to AM fungi. Moreover, bridges between plants formed by AM fungi can be established both by hyphal growth from one plant to another and by anastomosis where two hyphae of the same isolate fuse together and exchange nuclei. Giovannetti *et al.* (2001) demonstrated that anastomosis is very common, with fusion occurring every 2 mm of hypha. Thus, in our study, extensive functional mycorrhizal networks are expected to have established throughout the mesocosms.

Our experimental design also allowed us to tease apart any potential effects of soil diffusion or root-to-root contact from the effects of mycelial contact between plants, and the data suggest that transfer of signalling compounds via rhizosphere deposition is not the major pathway of below-ground plant-to-plant communication under the conditions of our experiment. Nevertheless, we cannot rule out that, in different natural conditions, pathways other than

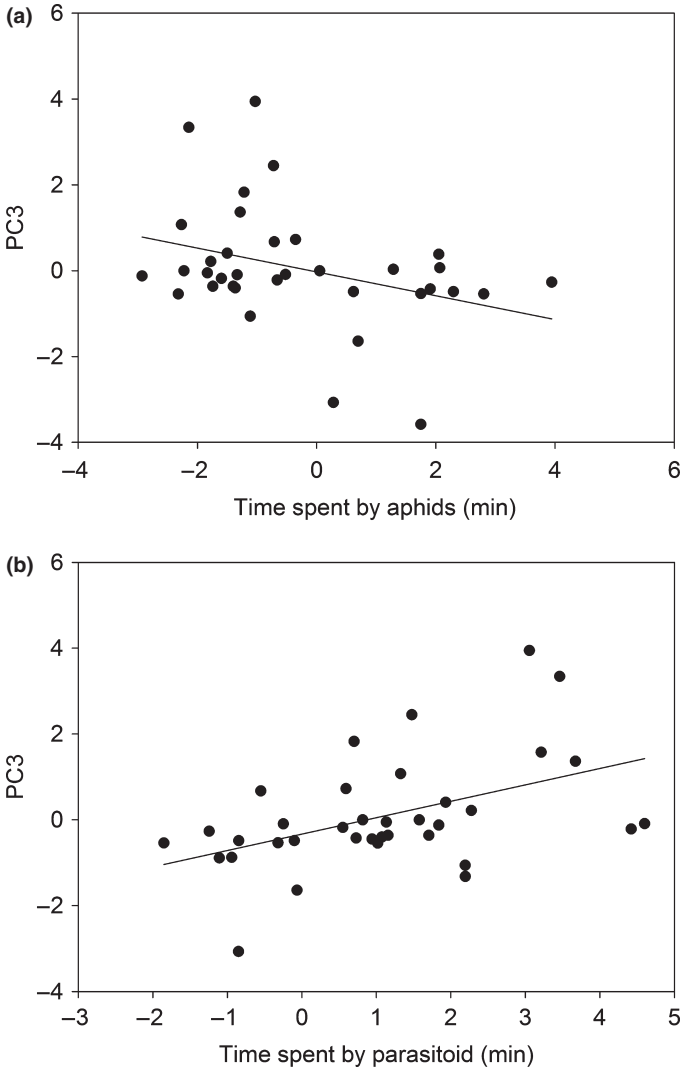

Figure 4 Relationship between principal component 3 (PC3) scores from principal component analysis on the correlation matrix of the amounts of electrophysiologically active volatiles produced by plants and insect host location response in olfactometer bioassays (time spent in area treated with headspace volatiles minus reagent blanks) of (a) pea aphid (Adjusted R-squared: 0.09; $P = 0.046$, $F_{1,34} = 4.29$) and (b) parasitoid wasp (Adjusted R-squared: 0.19; $P = 0.004$, $F_{1,34} = 9.38$).

AM fungal mycelia in the rhizosphere might also act as signal conduits. For example, there is a possibility that the signal might be transferred between plants in a liquid stream, or film layer, and it is also a possibility that formation of these might be greater in the presence of the fungal mycelia. Because the meshes used in our experiment were water permeable, it is unlikely that both of our independent methods of preventing hyphal connections (rotated 40 μm mesh core and non-rotated 0.5 μm mesh core) also prevented formation of liquid streams or film layers. From our experimental design, we therefore have confidence to attribute the signal transfer to fungal mycelium, or possibly some physical phenomenon associated with hyphal connection.

Signalling via common mycelial networks elicited emission of *V. faba* VOCs that are repellent to *A. pisum* aphids but attractive to a key natural enemy, the parasitoid wasp *A. ervi*. Our study shows that AM fungal networks provide a channel for interplant communica-

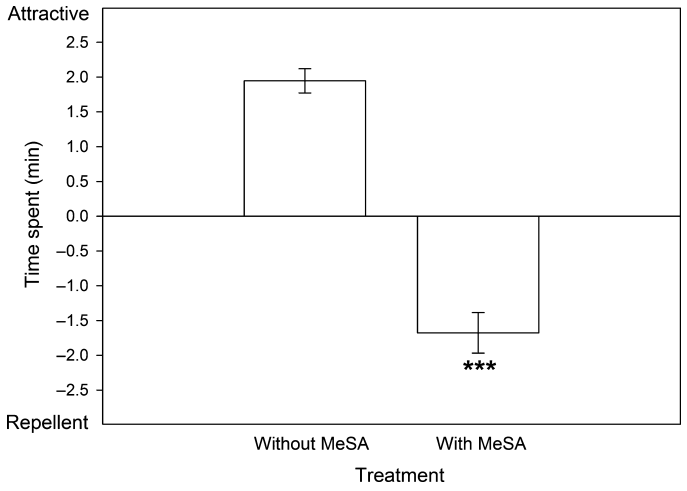

Figure 5 Effect of addition of methyl salicylate (MeSA) to previously attractive volatile organic compound extracts on pea aphid behaviour (± SE). ***Significant difference ($P < 0.001$, t = 10.9, d.f. = 6).

tion and enable plants to prepare for aphid attack with chemical defence mechanisms, without being in direct contact with the herbivore. This finding demonstrates that mycorrhizal fungal networks can function as a messaging system to neighbouring plants and trigger effects on organisms at different trophic levels. Such an early warning system may have profound consequences for the functioning of multitrophic systems, and highlights the need to consider linkages between above- and below-ground organisms (Wardle 2004), even when these organisms do not come into contact with each other.

Although we do not know the identity of the signalling compounds transported via the shared fungal network eliciting production of VOCs in uninfested plants, other work has shown that lipids such as triacylglycerols are actively transported through AM fungal mycelia (Bago *et al.* 2002). Identifying the compound(s) transported through the fungal network is outside the scope of this study, but is an important target for future research to elucidate the mechanisms regulating aphid-induced signal transport through AM fungal mycelia.

Most studies of common mycelial networks have focused on possible nutritional benefits to plants (e.g. Watkins *et al.* 1996; Simard & Durall 2004; Robinson & Fitter 1999). Our work provides evidence of an additional benefit from the formation of common mycelial networks. We do not know the extent to which communication of aphid-induced signalling molecules affect wider ecosystem properties, but there are clear possible benefits to both mycorrhizal plants and fungi. For example, it is known that infestation by aphids has profound impacts on plant allocation of carbon (Girousse *et al.* 2005) which may be detrimental for mycorrhizal fungi, and so it would be advantageous to the fungus if aphid populations were suppressed. Aphid populations can proliferate rapidly even following small-scale infestation of plants (Guerrieri & Digilio 2008), and so prevention of infestation of neighbouring plants is likely to be an effective mechanism to prevent large-scale infestations (Barto *et al.* 2012) and thus maintain a selective advantage to individual plants and fungi. Aphids have clumped distributions that fluctuate rapidly and thus it would be adaptive for plants neighbouring infested ones to prepare their defences before they are attacked.

The composition of VOCs released by leaves often differs between plants grown in the mycorrhizal and non-mycorrhizal condition (Guerrieri *et al.* 2004; Schausberger *et al.* 2012) so our experimental design, which used plants grown only in the mycorrhizal condition, enabled us to identify those compounds elicited specifically in response to common mycelial networks. The key VOC driving insect response to the plants in our system was identified as methyl salicylate. Several lines of evidence showed that release of methyl salicylate from leaves in plants connected to donors via mycelial networks underpins the behavioural responses of aphids in our experiment: First, methyl salicylate was one of the VOCs shown to elicit electrophysiological activity with the antennae of both pea aphids and parasitoids. Second, the quantities of methyl salicylate in headspace samples were significantly less from plants unconnected to donors compared to plants connected to donors and donors themselves. Third, methyl salicylate had the highest loadings with PC3 from the PCA, which was the principal component that correlated with behavioural responses of both aphids and parasitoids. Finally, addition of synthetic methyl salicylate to attractive plant headspace samples at the amount naturally present in repellent samples, made them repellent to aphids, providing clear experimental evidence that this compound is a key driver of aphid behavioural responses. Methyl salicylate has previously been shown to repel other species of aphids (Hardie *et al.* 1994) and attract parasitoids (Sasso *et al.* 2009), and is suggested to be a mobile signal that can be transported throughout plant tissue in phloem sap to induce systemic acquired resistance in tobacco plants (Shulaev *et al.* 1997; Park *et al.* 2007). Nevertheless, it remains a possibility that other VOCs (e.g. those with high loadings in PC3) may also have a role in affecting insect behaviour, either individually or by interaction with other VOCs.

It was found recently that some commercial cultivars of maize have lost their ability to produce herbivore-induced plant volatiles (Tamiru *et al.* 2011). It is therefore important to determine whether selective breeding of other important crops, such as beans, results in loss of their ability to either perceive aphid-induced signals from mycorrhizal fungi, or disrupt downstream signalling for production of VOCs. Moreover, manipulation of VOCs released by crops has considerable potential for pest control in the field (Xiao *et al.* 2012; Khan *et al.* 2010). Given the ubiquity of AM symbioses in herbaceous plants including most major crops (Smith & Read 2008), our data suggest a pressing need to determine the extent to which manipulation of common mycorrhizal mycelial networks can provide sustainable solutions to manage insect pests. The role of mycorrhizal fungi in mediating multitrophic interactions in agricultural ecosystems has largely been overlooked, but our findings suggest that there may be potential to develop fungal treatments to enhance crop protection.

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AUTHORSHIP

LG and DJ conceived the study, and together with TB and JP, acquired the funding and supervised the work; LG, DJ and ZB

designed the experiment; ZB performed volatile collections, bioassays and gas chromatography analyses; ZB and DJ analysed the data; CW performed electrophysiology; TB, MB, JC and ZB identified the volatiles; DJ, LG and ZB wrote the manuscript; TB and JP contributed to the revision.

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Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision

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Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision

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Flowering plants in Australia have been geographically isolated for more than 34 million years. In the Northern Hemisphere, previous work has revealed a close fit between the optimal discrimination capabilities of hymenopteran pollinators and the flower colours that have most frequently evolved. We collected spectral data from 111 Australian native flowers and tested signal appearance considering the colour discrimination capabilities of potentially important pollinators. The highest frequency of flower reflectance curves is consistent with data reported for the Northern Hemisphere. The subsequent mapping of Australian flower reflectances into a bee colour space reveals a very similar distribution of flower colour evolution to the Northern Hemisphere. Thus, flowering plants in Australia are likely to have independently evolved spectral signals that maximize colour discrimination by hymenoptera. Moreover, we found that the degree of variability in flower coloration for particular angiosperm species matched the range of reflectance colours that can only be discriminated by bees that have experienced differential conditioning. This observation suggests a requirement for plasticity in the nervous systems of pollinators to allow generalization of flowers of the same species while overcoming the possible presence of non-rewarding flower mimics.

Keywords: flower; bee; pollination; Australia; Gondwana

1. INTRODUCTION

In many flowering plant (angiosperm) species, the transfer of pollen from one flower to another is entrusted to animal vectors, such as insects and birds [1–3]. Typically, pollination vectors are attracted to flowers in search of floral rewards such as pollen and nectar, and in the process of visiting multiple flowers incidentally transfer pollen between compatible flowers. Plants typically attract and aid the orientation of important pollinators to their flowers by using relevant cues including olfaction [4], colour [5] and shape [6].

The relationship between angiosperms and animal vectors is very important. Plants that have rewarding flowers which are easily detected and discriminated will have an increased probability of distributing pollen to conspecifics, and thus successfully reproducing [5,7,8]. At the same time, animals that make correct foraging decisions will potentially collect more nutrition per unit time [9,10]. Visual ecology principles suggest that signal providers and/or signal receivers will evolve, within biological constraints, to optimize the efficiency of this biological partnership [1,11–14].

Insects are among the major pollinators of angiosperms. In particular, individuals of some hymenopteran species, such as honeybees and bumblebees, have a tendency to be 'flower constant', and will repeatedly visit one type of flower as long as these flowers continue to offer rewards [15,16]. It is probably that the reason why some individual pollinators exhibit flower constancy is a limitation on how working memory can learn and recall multiple flower types [15,17,18]. There are likely to be significant reproductive advantages for plants that can maintain flower-constant pollination vectors, since pollen is mainly delivered to conspecifics, rather than being randomly distributed, as would be the case for wind-pollinated angiosperms [15]. Thus, there are significant fitness benefits for angiosperms that have flowers which are easily discriminated by flower-constant pollinators.

It is known from both electrophysiological recordings [19] and behavioural testing [20] that honeybees have trichromatic colour vision based on ultraviolet- (UV), blue- and green-sensitive photoreceptors. This distribution of colour receptors is highly conserved in most other hymenopteran insects and is derived from a basal visual system that predates the evolution of angiosperms [19,21,22]. Colour discrimination should be optimal at wavelengths closest to the position where spectrally different photoreceptors overlap [19,23]. Thus, trichromatic hymenopteran pollinators are likely to have best discrimination for wavelengths close to 400 and 500 nm [19], and behavioural experiments on free-flying honeybees have confirmed this theory [24].

In a study that explored the potential ecological implications of pollinator vision on the colours of flowers that evolved in the Middle East (Israel), a very close fit was observed between the regions of the electromagnetic spectrum, where bees best discriminate colour information (400 and 500 nm), and the 'inflection points' at which flower reflection curves show the largest changes

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in the quantity of reflected radiation [25]. Interestingly, investigations in Israel have yielded one of the earliest fossil pollen records of angiosperms in the Hauterivian (*ca* 133 Ma) [26]. Palynological records from southern England also reveal evidence for the appearance of angiosperms around this time [27]. Studies comparing the distribution of flower colours suggest that visual ecological constraints from hymenopteran trichromats have been a major influence on angiosperm evolution throughout the Middle East and Europe [25,28,29]. Importantly, this evolution of flower colours has not been a coevolution as hymenopteran vision is phylogenetically ancient and predates the evolution of angiosperms [22].

The geological isolation of the Australian continent makes it an interesting target for studying angiosperm flower colour evolution. Australia has a very distinctive bee fauna, with emphasis on species of the family Colletidae, and relatively few Apidea; the latter representing less than 15 per cent of the known Australian species [30]. Importantly, Australia has been separated from other major continental land masses since at least the end of the Eocene epoch (*ca* 34 Ma) [31–35], and endured a period of isolation before coming into contact with scattered southeast Asian terranes in the Miocene, *ca* 25 MA [32]. While there is evidence that floristic interchange between the Northern and Southern Hemispheres did occur during the Mid- to Late Cretaceous, plant groups extending to each hemisphere readily differentiated into discrete provincial taxa [36]. The high-latitude Mesozoic position of Australia, its subsequent isolation and later northward drift have resulted in a high degree of endemism in the continent's flora (*ca* 6% of families, 22% of genera and over 80% of species are endemic [37]) with many lineages extending back to the Paleogene or Cretaceous [38]. It currently remains unclear, however, to what extent the evolution of flower colours on the Australian continent may have been shaped by the colour discrimination capabilities of hymenopteran pollinators, as has occurred in the Northern Hemisphere.

An important, related issue in the context of understanding flower evolution is the degree to which individual insects generalize similar colours [39,40]. Studies in honeybees [41–43], bumblebees [44–47], hawkmoths [48] and ants [49] reveal that individual insects learn perceptually similar colours very differently, depending on either absolute conditioning (learning a target colour in isolation), or differential conditioning (a target colour is linked to a reward while a distractor colour contains no reward). Differential conditioning leads to a significantly higher capacity to make fine colour discriminations [41,43–45,48,49]. Currently, the reason why insect pollinators demonstrate the behavioural plasticity to learn a target colour in different ways is unclear. One hypothesis is that pollinators initially need bandwidth to accept signals resulting from the natural variability in plant flower pigments [50]; but in a situation in which bees may encounter similarly coloured non-rewarding flowers, their visual system may need to have the ability to fine tune its responses to maximize the collection of nutrition [45,50,51]. These theoretical considerations suggest that flower variability from conspecific plants should lie within the range of colorimetric distances that pollinators can discriminate following differential conditioning, but otherwise generalize if receiving absolute conditioning.

Here, we test whether the evolution of flower colours in Australia fits the regions of the electromagnetic spectrum for which hymenopteran colour vision enables the best level of discrimination. We then use the dataset to understand the extent to which the degree of variability in colour signals produced by particular plant species matches the range of colour discrimination and generalization that has been observed in behavioural studies of important angiosperm pollinators.

2. MATERIAL AND METHODS

(a) *Is there a link between hymenopteran vision and Australian floral coloration?*

(i) *Data collection*

Australian native flowers were collected from Maranoa Gardens, Melbourne, Australia. Maranoa Gardens maintains a diverse collection of species from all over the continent. Species held in the collection are not selected on the basis of flower colour, but are selected by botanists to represent the diversity of Australian plants. Data collection was once per month from May 2009 to January 2010. During data collection, plants were chosen on the basis of a plant having more than three flowers present; otherwise plant selection was randomized. A UV photograph was taken of a flower from each plant using a digital UV camera (Fuji Finepix Pro S3 UVIR-modified charge-coupled device for UV imaging and fitted with a 105 mm f4.5 quartz UV-Nikkor lens and optically polished Baadar U-filter (325–369 nm half band width)) with calibrated UV-visible grey scales [52]. As UV rays are typically invisible to the human eye [53], this photographic representation enabled any different UV-reflectance areas of the flower to be identified and then measured with a spectrophotometer [54]. The spectral reflection functions of flowers were measured from 300 to 700 nm using a spectrophotometer (S2000) with a PX-2 pulsed xenon light source attached to a PC running Spectra Suite software (Ocean Optics Inc., Dunedin, FL, USA) and calibrated against a UV reflecting white $BaSO_4$ standard (Ocean Optics). A total of 111 plant species were sampled, each with three replicates. For data management, flower spectra will be contributed to the open access web portal Floral Reflectance Database to allow subsequent meta-analyses of flower reflectance data [55].

(ii) *Spectral measurement analyses*

Spectral data of flower reflectances were analysed using a previously established methodology, which has already shown that honeybee colour discrimination closely fits angiosperm colours that have evolved in the Northern Hemisphere [25,28]. For colours to be best discriminated by a visual system, the reflectance curves should rapidly change in the parts of the electromagnetic spectrum where spectrally different photoreceptors overlap [24,25,56]. We thus quantified the occurrence of inflection points where there was a change of greater than 20 per cent reflectance of radiation in less than 50 nm of the spectrum. The midpoint of a particular inflection point was determined within 10 nm bins, which allowed for the quantification of the wavelength at which spectral curves changed [25]. The data of the frequency of inflection points were plotted versus wavelength (λ) and compared with an inverse $\Delta\lambda/\lambda$

function that quantifies the regions of the visual spectrum in which honeybee vision can best discriminate spectral information [24].

(iii) *Colorimetric analyses*

Colorimetric techniques allow analyses of how flower reflectance curves are processed by the visual system of an animal. In this study, we used a hexagon colour space [57] to represent the distribution of flower colours that have evolved in Australia considering hymenopteran trichromat vision. The hexagon colour model was used in relevant previous studies [25,28], and makes no specific assumptions about colour opponent channels so is currently the most applicable general model of hymenopteran colour vision [57]. As mentioned previously, current evidence is that the photopigments underlying trichromatic vision in hymenopteran species are highly conserved, including for bee families native to Australia, and the photopigments are thus derived from a basal visual system that predates the evolution of angiosperms [21,22]. It is thus possible to model Australian bee colour perception using hymenopteran trichromatic models [58]. We modelled hymenopteran vision with spectral sensitivity peaks at 350 nm (UV), 440 nm (blue: B) and 540 nm (green: G) [21,22] using a vitamin A1 visual template [21,59–61].

For the colour hexagon model, the relative amount of radiation absorbed by each of the photoreceptors P (UV, blue (B), green (G)) was calculated by numerically integrating the product of photoreceptor absorption $S(\lambda)$, spectral reflectance $I(\lambda)$ and the illumination $D(\lambda)$ (equation (2.1)) at 10 nm steps from 310 to 650 nm. The variable K is used to normalize each of the photoreceptors to the illumination reflected from the background ([57,59]; equation (2.2)). The spectral quality of radiation was taken to be 6500 K, corrected for photon flux, to give a good match with typical daylight conditions for foraging insects [59,62]:

$$P(\mathrm{UV,B,G}) = K\int_{310}^{650} S(\lambda)I(\lambda)D(\lambda)\mathrm{d}\lambda \qquad (2.1)$$

and

$$K = \frac{1}{\int_{310}^{650} S(\lambda)I_{\mathrm{B}}(\lambda)D(\lambda)\mathrm{d}\lambda}, \qquad (2.2)$$

where $I_{\mathrm{B}}(\lambda)$ is the spectral reflectance of the background of green foliage.

The transduction of photoreceptor absorption (P) into receptor excitations (E) is given by

$$E = \frac{P}{P+1}. \qquad (2.3)$$

The receptor excitations (E_{SWS}, E_{MWS} and E_{LWS}) were plotted on orthogonal axes, each of unit length, and the colour of a flower was represented by the sum of the three vectors [57]. Coding is performed by two unspecified colour opponent mechanisms (x and y) and the output is given in equations (2.4 and 2.5) [57]:

$$x = \sin 60^{\circ}(E_{\mathrm{LWS}} - E_{\mathrm{SWS}}) \qquad (2.4)$$

and

$$y = E_{\mathrm{MWS}} - 0.5(E_{\mathrm{LWS}} + E_{\mathrm{SWS}}). \qquad (2.5)$$

Colour distance in the hexagon colour space can be determined by the Euclidean distance between loci [57]. These colorimetric values can be interpreted as perceptual distance using psychometric testing that has been conducted on bumblebees [50,63] and honeybees [64,65], the two main model systems for hymenopteran colour vision.

Australian native plant flower colour frequencies in colour space were determined with a radial grid of 10° sectors dissecting the distribution of colour loci, and the frequency of floral colour loci within each sector was counted as described in previous work [28].

(b) *Does variability between flowers of the same species explain why insect pollinators have behavioural plasticity for colour learning?*

Pollinator colour perception is dependent on individual experience (conditioning procedure) [41,43,44,48,50,51]. Using bumblebees as a model to map psychometric colour functions, and considering 70 per cent choices as the threshold for reliable recognition [24], it has been shown that discrimination can be divided into three cases: (i) colour distances less than 0.04 hexagon units are not reliably discriminated by bees, (ii) distances between 0.04 and 0.11 hexagon units are only discriminated if bees receive differential conditioning, and (iii) distances greater than 0.11 hexagon units are reliably discriminated even with absolute conditioning [50]. These three cases allow for the formulation of hypotheses about why the visual system of hymenopterans may have evolved the capacity for behavioural plasticity for colour discrimination.

H1: if the degree of variability in the pigmentation of flower colour for a particular plant species is less than 0.04 hexagon units then this variability is less than the perceptual threshold for bee colour vision. This case is a null hypothesis and would suggest that plasticity in pollinator colour discrimination is not linked to deal with the colour variability of plant flowers.

H2: if the degree of variability in the pigmentation of flower colour for a particular plant species is greater than 0.04 hexagon units but less than 0.11 hexagon units then this degree of variability in flower colour can only be discriminated by bees following differential conditioning. This case would suggest that bees generalize similar colours so long as flower stimuli present a reward (essentially a case of absolute conditioning), but if multiple non-rewarding flowers (e.g. non-rewarding mimics) were present in a foraging environment, an experienced forager can learn to make fine discriminations.

H3: if the degree of variability in the pigmentation of flower colour for a particular plant species is greater than 0.11 hexagon units, then this variability in flower colour is greater than the perceptual threshold for bee colour vision to reliably discriminate colours even with absolute conditioning.

Using this hypothesis-driven framework based on psychophysics testing, the colorimetry analyses method described above was used to determine the hexagon model colour difference between the data of the three flowers collected from each plant as sample 1 versus 2; 1 versus 3; and 2 versus 3 to produce one mean value of colour variability for each plant. This procedure was repeated for all 111 plants species, and overall colour variability was calculated as the mean (±s.d.) of the 111 values to represent variation in natural flower coloration as perceived by bee pollinators.

3. RESULTS

(a) *Is there a link between hymenopteran vision and Australian floral coloration?*

Flower reflection curves measured with a spectrophotometer allow for the quantification of a flower's spectral 'signature'. Figure 1 shows an example of spectral reflection curves of two native plant flowers, and slope midpoints, which allows for the determination of the relative frequencies with which the spectral signatures could be best discriminated by a colour visual system. Figure 2 plots the frequency of the slope midpoints relative to wavelength, and the inverted $\Delta\lambda/\lambda$ function [24], which shows how hymenopteran trichromats best discriminate colour signals relative to wavelength. The insert in figure 2 shows a comparative dataset from the Northern Hemisphere [25]. The high degree of similarity between these two datasets strongly suggests that a process of parallel evolution in response to similar ecological constraints has occurred. Interestingly, both datasets reveal an increase in the frequency of slope midpoints at wavelengths longer than 600 nm (figure 2), which is a part of the spectrum that hymenopteran trichromats discriminate very poorly [24,60].

To further understand how flower colours are perceived by hymenopteran pollinators, the loci of flower spectral reflectance curves were plotted in a hexagon colour space to model pollinator perception (figure 3*a*). This distribution was analysed as a frequency distribution using the sectors shown in figure 3*b*. The main figure (figure 3) shows the frequency of flower loci in the hexagon colour space sectors, and a comparative dataset using the same analysis technique for flowers from the Northern Hemisphere [28]. The similarity between the datasets suggests that hymenopteran colour vision has influenced flower colour in both Australia and the Northern Hemisphere in a similar way.

(b) *Does variability between flowers of the same species explain why insect pollinators have behavioural plasticity for colour learning?*

To understand the relationship between variability of the colour signals provided by different flowers of the same species, and the limits of behavioural plasticity for colour learning in pollinators, we also determined the mean in colour loci separation for flowers of different plant species (figure 4). The mean value of flower variability (0.054 ± 0.045 s.d. hexagon colour units) falls in a range of colour discrimination that is consistent with the hypothesis H2, i.e. that colours of flowers from the same plant species are only reliably discriminated by bees that have experienced differential conditioning. A statistical analysis of the colour variability compared with the set threshold value of 0.04 hexagon units is significant from chance (one-sample *t*-test, $t_{110} = 3.276$, $p < 0.001$); showing that the degree of variability in natural flower colours is potentially an important problem that the visual system of pollinators has to overcome.

4. DISCUSSION

Colour is a major cue for how pollinators find flowers, and the colour perception of pollinators may influence which flower colours evolve more frequently. The current

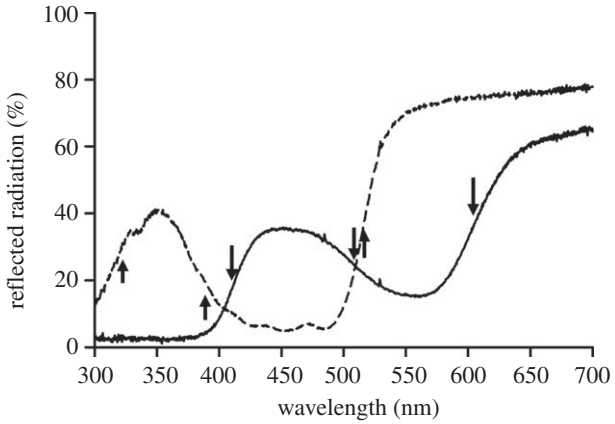

Figure 1. Example floral spectral reflections of two Australian native flowers (endemic): *Hibbertia scandens* (dashed lines, human yellow) and *Pandorea jasminoides* (solid line, human pink). The arrows show the slope midpoints [25].

study sampled a range of Australian native plant flowers and showed that the most frequent occurrence of changes in the reflectance curves fits with the regions of the electromagnetic spectrum 400 and 500 nm, where hymenopteran trichromats best discriminate spectral differences (figure 2). These data strongly suggest that hymenopteran pollinators have been a major driving force in the evolution of angiosperm flower coloration in Australia. This finding agrees with data suggesting that the majority of Australian native hymenopteran species are polylectic [30], and thus their visual capabilities can potentially influence the evolution of a wide range of flowering plants.

Another possibility that could explain the very close fit of data in figure 2 is that other potentially important pollination vectors, such as birds and/or butterflies, might also possess enhanced spectral discrimination in the 400 and 500 nm regions of the electromagnetic spectrum. The visual system of birds typically contains four spectral classes of single cones that contribute to colour discrimination [23,66,67]. While there is variability in single cone spectral sensitivity in birds [14,66,67], of the 14 avian orders tested to date, birds fall into two main groups [66]. The violet sensitive (VS) group has VS ($\lambda_{max} \sim 400$–430 nm), short wavelength sensitive (SWS; $\lambda_{max} \sim 450$–480 nm), mid wavelength sensitive (MWS $\lambda_{max} \sim 530$–550 nm) and long wavelength sensitive (LWS $\lambda_{max} \sim 600$–620 nm) spectral sensitivities considering ocular filtering, while the ultraviolet sensitive (US) group has US ($\lambda_{max} \sim 360$–380 nm), SWS, MWS and LWS spectral sensitivities. The visual behaviour of the pigeon has been well studied, and while not a major pollination vector, the $\Delta\lambda/\lambda$ function for the pigeon has been measured and is a representation of the visual capabilities of the VS group of birds. Behavioural data for wavelength discrimination by pigeons show minima at 460, 540 and 600 nm [68,69], and qualitatively similar values have been empirically measured for the hummingbird [70]. Thus, the visual system of VS birds does not correspond well with the high frequency of flower inflection points in figure 2. The budgerigar has photoreceptor peak spectral sensitivities at 365, 462, 513 and 581 nm when considering the effects of oil droplet filtering [71], and is a representative model of the US-type avian

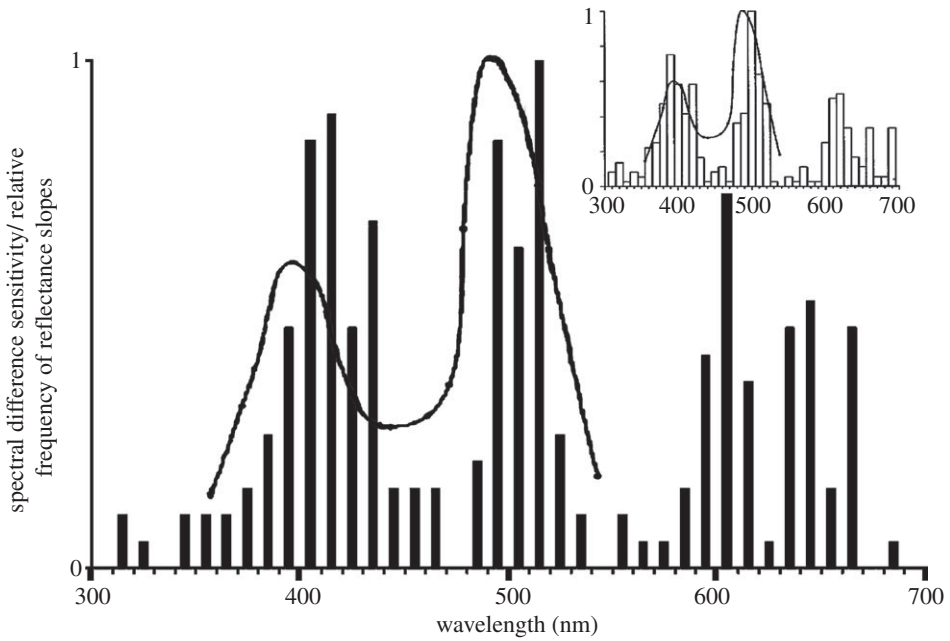

Figure 2. Relative frequency of Australian native plant flowers ($n = 111$ flowers; highest column $n = 19$ step functions) having reflectance curve slope midpoints (black bars) compared with the inverse $\Delta\lambda/\lambda$ wavelength discrimination function (solid curve) of the honeybee [24]. The insert shows data for flowers from Israel ($n = 180$ flowers; highest column $n = 36$ step functions) from a previous study that used the same methodology [25]. Australia is geologically well separated in space and time from the rest of the world and has a distinctive bee fauna [30]; the data suggest parallel evolution of many angiosperm flower signals to be best discriminated by hymenopteran trichromats.

visual system with theoretical wavelength discrimination minima based on modelling and behavioural testing at about 416, 489 and 557 nm [71]. While the 416 and 489 nm minima approximately match the spectral data in figure 2, there is no corresponding peak at around 557 nm in the flower reflectance data (figure 2), but biochemical and phylogenetic constraints should allow for these types of reflectance curves if there was sufficient evolutionary pressure [36,72], and these reflectance curves do exist at low frequencies (figure 2). This suggests that birds having a US visual system also do not match the data in figure 2.

Unlike the phylogenetically conservative spectral positions of colour photoreceptors in hymenopteran trichromats [21,22], the spectral properties of different butterflies show a large degree of diversity [73–75] and can be trichromatic, tetrachromatic or pentrachromatic [73,75]. Both molecular tuning of opsin genes [73,76] and pigment filtering [77] suggest that butterfly spectral sensitivity differences evolved relatively rapidly, leading to a large degree of diversity of colour capabilities in these insects [73]. It is thus unlikely that butterfly pollinators, when considered as a group, could explain the fit of data in figure 2, because the colour discrimination capabilities of these insects would, in some cases, predict very different flower colours. For example, the $\Delta\lambda/\lambda$ function has been measured for the butterfly genus *Papilio* and reveals three minima at approximately 430, 480 and 560 nm [78], and these minima do not fit with the data for most frequently evolving flower colours (figure 2). Another group of potentially important pollinators is flies [79]. While some flies such as *Musca* do have trichromatic spectral sensitivities close to those of hymenopteran trichromats [21,80], fly spectral sensitivities can be readily shifted with molecular manipulations to the opsin sequence [81], suggesting different fly species do not possess colour vision that is as conserved as hymenopteran trichromats [21]. In addition, recent work on fly pollination suggests that olfaction is the main cue used by flies to discriminate between flowers, while colour is not an important cue for these pollinators [82]. It is unlikely that flies are the major driver behind the evolution of flower colours for two other main reasons: (i) as far as is currently known from behavioural experiments on flies, their colour perception is relatively rudimentary and is mediated by simple categorical colour discrimination (i.e. spectral differences are only perceived as either 'same' or 'different' to a training stimulus, depending on whether they lie inside a limited number of colour categories) [82,83]. Thus, there is currently a paucity of behavioural data on flies to support that these insects do discriminate colour information in a way that would be the major driver of flower evolution; and (ii) while there is evidence that some flies such as hoverflies do exhibit flower constancy [79], colour cues do not appear to be a factor in flower-constant behaviour in flies and these insects choose randomly between morphs varying in colour [79]. Consequently, compared with social hymenopteran pollinators [15], flies are probably less-efficient pollinators of angiosperms, although more work on this topic would be of high value.

In summary, neither the colour discrimination capabilities of birds, nor butterflies, match the close fit of flower reflectance data to hymenopteran vision at 400 and 500 nm (figure 2). In addition, fly colour discrimination capabilities and flower-constant behaviour for colour cues appear poor in comparison with hymenopteran trichromats, suggesting hymenopterans are likely to be more influential drivers of colour evolution. However, the evidence of relatively fine colour discrimination

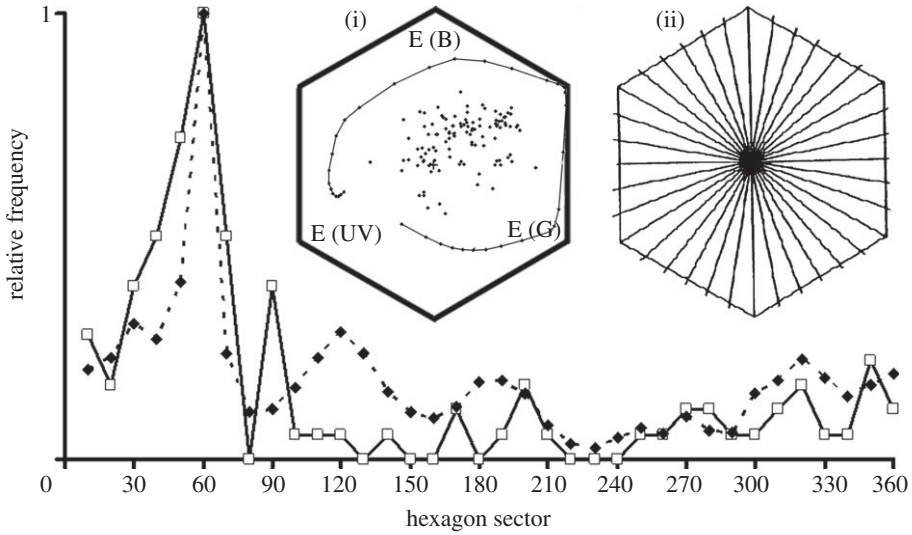

Figure 3. Relative frequencies of Australian native plant flower distributions (main figure solid line) compared with a previous study on flower evolution in the Middle East (dashed line, [28]). Data are plotted considering the visual system of hymenoteran trichromats in a hexagon colour space (see insert (i)), and the frequency with which flower colour loci were distributed in 10° sectors (see insert (ii); 0° is at the 12 : 00 angle in the colour hexagon and angles on the abscissa of the main figure read clockwise from 12 : 00) of the colour space. A similar distribution of flower colours has evolved in Australia and the Middle East, despite a very long geological separation of these study sites.

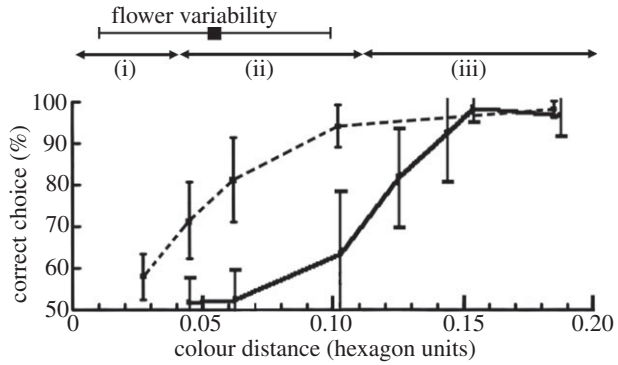

Figure 4. Colour discrimination (data from Dyer [50]) of stimuli by bumblebees (*Bombus terrestris*) considering either differential- (dashed line) or absolute- (solid line) conditioning relative to the perceptual distance in a hexagon colour space for hymenopteran colour vision (mean ± s.d.). Horizontal bars show colour distance for which: (i) colours are below threshold for either differential or absolute conditioning, (ii) colours are only reliably discriminated by bees that have experienced differential conditioning, or (iii) colours are reliably discriminated by bees even with only absolute conditioning. The variability in Australian native flowers of the same species ($n = 111$ flowers; mean ± s.d.) lies within the colour discrimination region where similar colours are only discriminated if bees have received differential conditioning (see text for statistics).

in birds and butterflies at longer wavelengths may explain the increased frequencies of some plant flowers having inflection points at these wavelengths (figure 2), although the more likely possibility is that plant material often reflects increasing amounts of radiation at wavelengths greater than about 600 nm [25,28]. Interestingly, for bird-pollinated plants, there is some evidence of both flower colours evolving spectral signals to maximize discrimination by birds [84], and/or birds evolving different spectral sensitivities to enhance discrimination of certain flower colours [14]. This would be an interesting topic to explore considering plant flowers that are either exclusively bird, or insect-pollinated.

To interpret the current finding for Australian angiosperms in relation to a previous study that reported a link between hymenopteran colour vision and angiosperm evolution in Israel [25], it is important to consider the timeframe for geological isolation of these land regions. Australia fully separated from other major land masses around 34 Ma [31–34], but terrestrial links were tenuous before total isolation. This timeframe, and the continent's shift from high-latitude moist to mid-latitude dry climates through the Cenozoic, imposed very different pressures on the evolution of Australian plants. An alternative explanation for the similarity in the flower reflectance datasets (figures 2 and 3) is that angiosperms may have evolved particular spectral properties prior to the development of a major sea barrier, or possibly island hopped in the periods following marine separation [85], and that these early plants then had phylogentic or biochemical constraints that subsequently influenced flower evolution in Australia. Indeed by the Aptian (125–112 Myr ago), angiosperm pollen and macrofossils occur in Australia [86,87], and in roughly, coeval strata in South America, Antarctica and New Zealand [88–91]. While the pollen record for the Late Cretaceous indicate the appearance of several typical austral taxa [92] and potential sister-group relationships of these taxa to Northern Hemisphere genera [93], the plant groups extending to each hemisphere readily differentiated into discrete provincial taxa [36]. This resulted in Australia, New Zealand and Antarctica acquiring a distinctive austral flora by the end of the Mesozoic, whose genetic signature persists in the region's modern vegetation. The few fossil flowers recorded from the Cretaceous of southern Gondwana are diminutive, 'non-showy' forms with short bracts, bracteoles, tepals or petals [86,91,92]. None of these early austral fossil flowers reveals evidence of colour, which is consistent with other

evidence suggesting that early angiosperms did not have salient colour signals [94]. However, further evidence on the potential pigmentation of early angiosperms would be of high value for more fully understanding the initial stages of flower colour evolution. Plant groups with elaborate and showy flowers (e.g. *Myrtaceae*, *Cunoniaceae*, *Sapindaceae*, *Ericaceae*, *Bombacoideae*, *Loranthaceae*, *Sterculiaceae*, *Elaeocarpaceae*, *Fabaceae*, *Rutaceae* and *Asteraceae*) make stepwise appearances into Australasia from the early Paleogene to the early Neogene, partially spanning the terminal breakup and isolation of Eastern Gondwana [95,96]. Thus, even though angiosperms reached Australia prior to the continent's total isolation, the very large time scale suggests that flower colour evolution in Australia was probably independent to that in the Northern Hemisphere. This evidence suggests angiosperms independently evolved spectral signals and these signals were not constrained by phylogenetic contrasts of plant pigments. This conclusion is also evidenced by the data in figures 2 and 3, which shows that while certain flower colours are more frequent in nature, a wide range of flower colours can be potentially generated by plants both in the Northern Hemisphere [72] and Australia.

The evidence that angiosperms evolved spectral signals in Australia that are parallel to the evolution of flowers in the Northern Hemisphere to suit hymenopteran colour vision (figure 2) is also reflected in the similar distribution of flower colours in a colour space characteristic of bee colour perception (figure 3). Interestingly, for both study sites, there is a considerably higher frequency of flower loci in the blue to green sections (around the 60° sector) of the colour space, while loci representing pure UV and UV to blue colours are relatively rare [29,60]. One possibility for this scarcity of certain flower colours is owing to theoretical considerations that colour constancy mechanisms in bees [59,63] work poorly for UV to blue-coloured flowers owing to overlap of bee colour photoreceptors in the UV region of the spectrum [59,60], which has some empirical support from behavioural experiments [29,63].

A second important finding of the current study is that the within-species variability in flower colour is in a range that bee colour vision can only discriminate if the bees have received differential conditioning to stimuli (figure 4). This suggests that bee colour discrimination initially generalizes similar colours so that there is sufficient bandwidth to tolerate the natural variability in potentially rewarding target flowers. However, the visual system of hymenopteran insects has plasticity to learn, with differential conditioning, to make relatively fine colour discriminations and thus allow experienced individual pollinators to avoid non-rewarding mimics that are similarly coloured to rewarding model flowers if required to do so [51]. This finding helps explain how mimic (i.e. non-rewarding) flowers such as some orchids can initially gain sufficient pollinator visits to successfully reproduce despite not offering rewards, but in many cases, mimic plants remain relatively rare [97–99]. For future work, it will be of high value to understand if such non-rewarding flower species may have evolved to share similar spectral properties to rewarding flower species.

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Extreme Reproduction and Survival of a True Cliffhanger: The Endangered Plant *Borderea chouardii* (Dioscoreaceae)

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Abstract

Cliff sides are extreme habitats, often sheltering a rich and unique flora. One example is the dioecious herb *Borderea chouardii* (Dioscoreaceae), which is a Tertiary, tropical relict, occurring only on two adjacent vertical cliffs in the world. We studied its reproductive biology, which in some aspects is extreme, especially the unusual double mutualistic role of ants as both pollinators and dispersers. We made a 2-year pollination census and four years of seed-dispersal experiments, recording flower visitors and dispersal rates. Fruit and seed set, self-sowing of seeds, seedling recruitment, and fate of seedlings from seeds sowed by different agents were scored over a period of 17 years. The ants *Lasius grandis* and *L. cinereus* were the main pollinators, whereas another ant *Pheidole pallidula* dispersed seeds. Thus ants functioned as double mutualists. Two thirds of all new seedlings came from self-sown seeds, and 1/3 was dispersed by ants, which gathered the seeds with their oil-rich elaiosome. Gravity played a minor role to dispersal. Both ant dispersal and self-sowing resulted in the same survival rate of seedlings. A double mutualism is a risky reproductive strategy, but *B. chouardii* buffers that by an unusual long–term demographic stability (some individuals exceed 300 years in lifespan) and its presence in a climatically very stable habitat, inaccessible to large herbivores. Such a combination of traits and habitat properties may explain the persistence of this relict species.

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Introduction

Ants are ubiquitous in nature, playing key ecological roles, not only in tropical and temperate ecosystems [1], but also in harsh environments like deserts and alpine habitats [2]. Two of their ecosystem functions are pollination and seed dispersal.

Ants are frequent floral visitors [2], but are often regarded as inefficient pollinators because of their small body size, short foraging range, and secretions from their metapleural glands, which may reduce pollen viability (''the antibiotic hypothesis'') [3,4]. However, several reports demonstrate the importance of ants as pollinators, e.g. in a population of an alpine plant, Gómez and Zamora [5] showed that one of the flower–visiting ant species enhanced plant female fitness more than all the 39 winged insect visitor species together. The high frequency of ant visits and their presence during the entire flowering period may be reasons for the pollinatory success of this single ant species [6,7]. Generally, ant pollination may be most common where abiotic conditions for flying insects are adverse, e.g. in mountains and deserts [2,5,7,8].

Despite that ants are well known as seed predators or harvesters [9], they also play an important role as seed dispersers. Seed dispersal by ants is known from at least 3,000 plant species but may be found in four times as many [10]. It has evolved independently in more than one hundred lineages, which subsequently diversified more than their non–ant–dispersed sister lineages [10]. This accelerated diversification rate was kicked off by a key innovation, the elaiosome, i.e. a small food body attached to seeds, which attracts ants [10]. The elaiosome is lipid–rich and nitrogen–poor. Typically, ants harvest the seed with its elaiosome and carry it back to the nest, where they bite off the elaiosome and feed it to their larvae. Afterwards, the ''garbage'', that is the seed without elaiosome, is deposited either inside the nest or outside in a refuse pile [10]. This behaviour may enhance plant fitness by moving seeds to seed predator–free and nutrient–rich sites suitable for germination or to a seed bank during periods of abiotic stress, reducing intraspecific competition [11,12]. Ants are, however, probably mediating the shortest seed flow of any animal disperser, viz. only 0.01–77 m [13].

For most plants, the pollinator and seed–disperser fauna differ from each other [14]. However, in habitats poor in animal diversity, such as deserts, islands, and mountains, plants may use the few resources available, and consequently, evolve towards double mutualism, i.e. to use the same animals as both pollinators and seed dispersers. A few examples are known, e.g. several island plants have lizards, birds or flying foxes as their double mutualists [15–17].

In mountains, cliff sides constitute ''ecological islands'', and they are among the resource–poorest habitats in the world [18]. In

recent years, they have received increasing attention by ecologists, [18,19]. Besides their steep orientation, cliff sides have ecological characteristics that distinguish them from other habitats: low availability of nutrients, very limited space for root development and scarce possibilities for biotic recruitment. Species able to live under such conditions, however, may be protected against climatic extremes (for example in deep canyons), large herbivores and most human effects. The fact that rock plants often are small and long–lived, but make up stable populations, suggests their rate of recruitment and mortality is very low [20,21]. However, several aspects of their life history are enigmatic. For example, how do rock plants get their seeds dispersed to safe crevices, avoiding that their populations after a few generations "slide" down the cliff side and go extinct?

Here, we address this question by studying the role of ants to the pollination and seed dispersal of one of the most ancient and endangered European plants, *Borderea chouardii* (Gaussen) Heslot (Dioscoreaceae) [22,23]. It is a small, strictly cliff–growing or rupicolous plant, occurring on shady, vertical limestone cliffs and overhangs in the central Spanish Pyrenees. The species belongs to a small dwindling element of relicts from a long gone Tertiary tropical flora, and it has the highest conservation priority in Europe (European Commission, Environment: Habitats Directive; Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora). However, the life span of its individuals is very unusual, being one of the longest ever recorded for any non–clonal herb, viz. >300 years [21]. Given the difficulties to conduct standard studies in the habitat of the plant, we accumulated detailed observations and carried out experiments in both the field and the lab for up to 17 years, to demonstrate a double mutualistic interaction between the plant and its pollinating and seed–dispersing ants. Finally, we discuss the role of these ants in relation to the *B. chouardii*'s astonishing long–term persistence.

Materials and Methods

The Plant

Worldwide, *Borderea chouardii* is known from one single population (Fig. 1A), located 850 m a.s.l. in the Spanish Pyrenees [24]. It was discovered only 60 years ago, probably due to its occurrence in a topographically complex and inaccessible area. Here its entire habitat covers a few thousand m^2. The plant grows on two vertical cliff walls and even upside down under the "ceiling" of a short cave without receiving any direct sunlight. The walls are stable, and only a few downfalls of stone chips have been recorded during the study period 1995–2011. A demographic monitoring project of a large sample of plants was initiated in 1995, using scaffolding and climbing gear [21].

The species is a small, dioecious geophyte of <2 g in individual biomass, with twice as many male as female plants. The tuber grows in small crevices in the wall without any vegetative propagation. Male plants produce more and smaller leaves, and flower at an earlier age and more profusely than females (Fig. 1B,E; M. B. García *unpublished data*). Both sexes have small, green flowers with tiny amounts of nectar. The ovary has six ovules. Floral pedicels are often close to the rocky wall (Fig. 1C, D) and once fertilized, female flowers turn towards the wall. The pedicel of ripening fruits may even elongate pressing the fruits into a crevice, where the seeds become released (Fig. 1E). This is termed self-sowing [21]. Seeds are brown, ovoid shaped, about 3 mm long, and have a tough oily coat, which becomes very dense at the apex. This coating and the dense apex function as an elaiosome (Fig. 1F).

Pollination and Reproductive Success

In order to identify flower visitors of *B. chouardii*, we spent 76 hours observing plants for flower–visiting insects, viz. 61 hours and 15 hours in 2008 and 2009, resp., or 53 (69%) and 23 hours (31%) observing males and females, resp. These focal plants were chosen randomly within the narrow vertical zone on the cliff wall of the population. Flower–visitation observations were made from 17–30 May, covering the entire flowering period. We did 397 censuses, each lasting 10–15 min at both groups and solitary plants. Gender, and numbers of open flowers per plant and flower visits by insects were recorded. Whenever in doubt about taxonomic status of a visitor it was sampled for later identification. *t*-tests were used to compare the frequency of visits to male and female plants and flowers. The likelihood of wind pollination was assessed by placing microscopic slides with glycerol on the walls 20 cm from a flowering male, and later inspecting slides for pollen.

Annually from 1995 to 2011, fruit set (the ratio of numbers of fruits : flowers), and seed set (the ratio of numbers of seeds : 6 ovules in ripening fruits) were estimated [21]. Fruit ripening happened in September and seeds were either dispersed by ants (*A*), gravity (*G*) or through "self-sowing" (*S*) (Fig. 2).

Seed Dispersal

Self-sowing was estimated between 1995–2011 as the percentage of ovaries growing within crevices. The rest of the fruits ripe mostly in contact with the rocky surface, where the dry capsules open and may contain up to 6 seeds. During the fruit–ripening period of *B. chouardii*, three ant species were observed at the study site: *Pheidole pallidula*, *Lasius grandis* and *L. cinereus*. In order to determine the role and importance of ants as seed dispersers, and given the difficulty of monitoring *in situ* seed dispersal and seedling recruitment, we gathered information from a set of experiments.

Experiment I. We made an *in situ*–"cafeteria" experiment to test the hypothesis that the ants we observed in the population were seed dispersers, and that the elaiosome was the unit of attraction (Fig. 1F). The experiment was commenced at the onset of the natural seed release in the population. Forty vials (1 cm wide, 4 cm deep) were glued to the cliff wall (Fig. 1G) and in each vial, we placed either (a) six seeds with elaiosome (2008 and 2011), (b) three seeds with elaiosome + three elaiosomes + three seeds without elaiosome (2009; for protocol details see [25]), or (c) one open fruit containing six non-shed seeds (2010). Every 5–10th day during six weeks, vials were inspected and numbers and kinds of removed items were scored. In 2008 and 2009, a few vials were lost or got filled with rainwater, leaving 37 and 35 vials for analysis, respectively. Generalized linear models (*glm* function, R Core Development Team 2011) were used to test for the preference of items by ants.

Experiment II. We also wanted to know if seeds of *B. chouardii* were particularly attractive to ants compared to other species with or without elaiosome. Given the difficulty to find nests in the population, in September 2008 we used 12 natural nests *P. pallidula* (the only species observed to remove seeds from *in situ*–vials; Fig. 1H) for a food–choice experiment in another location. Seeds of four plant species were offered to the ant: (1) *B. chouardii*, (2) its congeneric, the scree plant *B. pyrenaica* (its seeds have an oily coating too but no distinct elaiosome at their apex, (3) the rupicolous *Sarcocapnos enneaphylla* (seeds with elaiosome and co–occurring with *B. chouardii*), and (4) the partially rupicolous *Silene acaulis* (seeds without elaiosome and not co–occurring with *B. chouardii*). A group of four seeds (one of each species) was placed 10 cm from the entrance of each nests. During 10–min intervals of observation, the behaviour of *P. pallidula* workers to the presence of seeds was recorded. When leaving their nest, ants always had the

Figure 1. ***Borderea chouardii.*** (A) Topography of the habitat (Sopeira, Spanish Pyrenees), (B) flowering male plant, (C) flowering female plant with a visiting ant (*Lasius*), (D) male flowers with a visiting ant (*Lasius*), (E) female plant with two fruits, one at a crevice in the cliff wall (self sowing), (F) seed with elaiosome coating, (G) vial containing seeds *in situ*–for cafeteria experiments (Experiment I, see Materials and Methods), (H) ant removing a seed (Experiment I), and (I) tuber with old leaf scars marked with a red coloration, each dot corresponds to one year, and a ruler shows its size in mm.
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possibility to choose among all four species of seed, because if an ant removed a seed, it was immediately replaced. Possible ant responses were: "not removed" (either not interested in any seed, or seed examined but not removed), and "seed removed". Total number of ant responses was 514. Generalized linear mixed models (GLMM) were used to model the preference of ants to specific seeds. "Nest" was treated as a random factor, and the *lme4* function in R was used [26].

Experiment III. Finally, we tested how the two ant genera (*Pheidole* and *Lasius*) treated seeds of *B. chouardii*. Since it is not possible to observe the handling of seeds within nests on the wall, we studied this experimentally in the lab. A total of 30 seeds of *B. chouardii* were placed in front of the entrance to five artificial nests of *Pheidole pallidula*. We recorded if seeds were introduced to the nest, and one week later if they still had the elaiosome, were without elaiosome or were destroyed by predation.

In November 2010 after the fruiting season of *B. chouardii*, we carried out one further *ex situ*–experiment with the two *Lasius* species. Once a week, three artificial nests of *L. grandis* (young colonies: 1 queen +10–12 small workers) and *L. cinereus* (>100 workers +50–100 larvae) were offered six seeds of *B. chouardii*, and additionally, fed an artificial diet [27]. In mid–December, we had

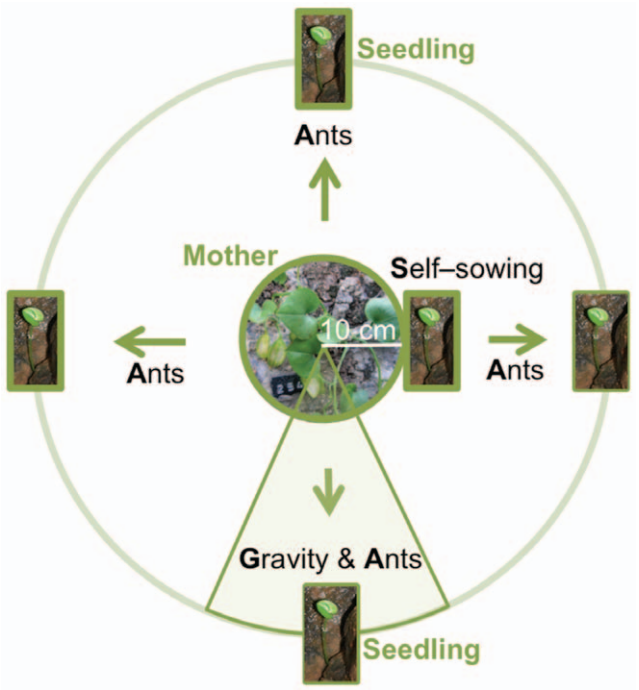

Figure 2. Seed–dispersal modes of *Borderea chouardii*. Dispersal by gravity (G) is assumed to take place within a circular section of 45° below a mother and >10 cm away from the mother. Dispersal by ants (A) takes place in all directions and >10 cm away from the mother, and self–sowing (S) is restricted to a circular area of a radius of 10 cm and the mother plant as its center.
doi:10.1371/journal.pone.0044657.g002

to let the nest hibernate, and the six nests were placed outdoor. In mid–February, they were returned to the laboratory (18–22°C). Position of seeds (out/inside the nest) and condition (elaiosome present/absent) were scored immediately before hibernation, and four weeks after hibernation period. Hibernating larvae began to develop normally after hibernation and to pupate.

The Environmental Service of the Regional Governement of Aragón gave the permit to do *in situ* and *ex situ* experiments involving seeds. The regional government is the responsible authority for the recovery plan implemented in 1995.

Successful Seed Dispersal and Survival of Seedlings

During 1995–2011, we studied the relative importance of different seed dispersal modes by recording the position of all new seedlings (1-year old) in the monitored area, and estimated survival probability. These represent successful dispersal events. We hypothesized that dispersal could take place in three ways: by self-sowing (*S*), ant (*A*), and gravity/rain (*G*). *S* included seeds dispersed <10 cm, i.e. within the circumference of the pedicels of a female plant (same crevice or a close one reachable by fruiting pedicels; see Fig. 1E). *A* included seeds dispersed >10 cm from nearest female plant but not directly below a female. Finally, *G* included seeds dispersed >10 cm and directly below the nearest female (Fig. 2). In order to distinguish between *A* and *G* in the field, we took into account the direction from each seedling to nearest female. If a female was growing directly above the seedling (within a circular section of ±23°) and being >10 cm away, the dispersal was scored as *G*, if not as *A* (Fig. 2). Nevertheless, ants can also move seeds downwards, and thus a small fraction on *G*-seedlings could actually come from *A*-. Dispersal rates were adjusted accordingly (see the results section).

The survival probability of all seedlings recorded over 17 years of monitoring was compared among different dispersal modes (*S, A, G*) by generalized linear models (*glm* function in R, binomial distribution).

Results

Pollination and Reproductive Success

Habitat and habit of *B. chouardii* are shown in Fig. 1. Population sex ratio, i.e. the numbers of individual male to female plants, was 2.2 ($N = 346$; Table 1). Male and female plants had 44.4±47.1 (mean ± SD; $N = 239$ plants, range 1–244) and 4.4±3.7 ($N = 107$ plants, range 1–23) simultaneously open flowers, respectively. Thus open male flowers were ($2.2 \times 44.4/4.4 =$) 22 times as frequent as female flowers in the population. Male plants received the same number of visitors but more visits (plant^{-1} hour^{-1}) than females (*t*–test (ln(x +1)–transformed data): $t = 1.41$, $P = 0.16$ (visitors); $t = 2.51$, $P = 0.01$ (visits); $N = 397$ plant visitor/visit census), whereas individual male and female flowers had the same visitation rate (visits flower^{-1} hr^{-1}) (*t*–test (ln(x +1)–transformed data): $t = 1.71$, $P = 0.09$; $N = 397$ floral visit census).

During the entire flowering season (17–30 May) in 2008 and 2009, we observed a total of 58 flower visitors (Table 1). Seventy percent were ants: *Lasius grandis* (59% of all ant records), *L. cinereus* (11%), *Camponotus cruentatus* (11%) and unidentified Formicidae species (19%) (Fig. 1C,D). Besides ants, a Collembola species (seven visitors), a parasitic Hymenoptera species (five visitors), a Coleoptera species (two visitors), and a Neuroptera species (one visitor) were observed in the flowers. Ants constituted 82% of all visitors to female flowers because they received less visits from non–ants. In the wind–pollination experiment, no *B. chouardii* pollen at all were found on any microscopic slide ($N = 10$ slides).

Across 17 years, mean fruit set was 82.8% ±8.5% (average ± SD; $N = 3{,}287$ flowers, range 59%–98%; Fig. 3), but fruit set has been declining ($R^2 = 0.30$; $P = 0.02$). In fruits seed set was 74.1% ±6.2% (average ± SD; $N = 2{,}761$ fruits, range 60%–82%; Fig. 3), and it also declined significantly ($R^2 = 0.61$; $P = 0.001$).

Seed Dispersal

Only 8.4% ±3.9% of the 2,568 fruits examined between 1995–2011 were self-sowed. Therefore, most seeds produced in the population were eventually released on the air unless harvested by ants or retained in crevices when rolling down by gravity.

Experiment I. In September during fruit ripening, only one ant species (*Pheidole pallidula*) was observed to remove seeds from

Table 1. Flower visitation of *Borderea chouardii*.

	Male	Female	Male : female
Observation time (hrs)	53	23	2.3
No. observed plants	239	107	2.2
No. flowers	8329	456	18.3
No. flowers/plant	44.4	4.4	10.2
Obs. time (min)/plant	13.2	12.9	1.0
Total no. visitors	47	11	4.3
Total no. ants	33	9	3.7
No. visitors/plant/hr	1.0	0.8	1.4
No. visits/plant/hr	3.7	1.0	3.6
No. visits/flower/hr	0.1	0.3	0.3

doi:10.1371/journal.pone.0044657.t001

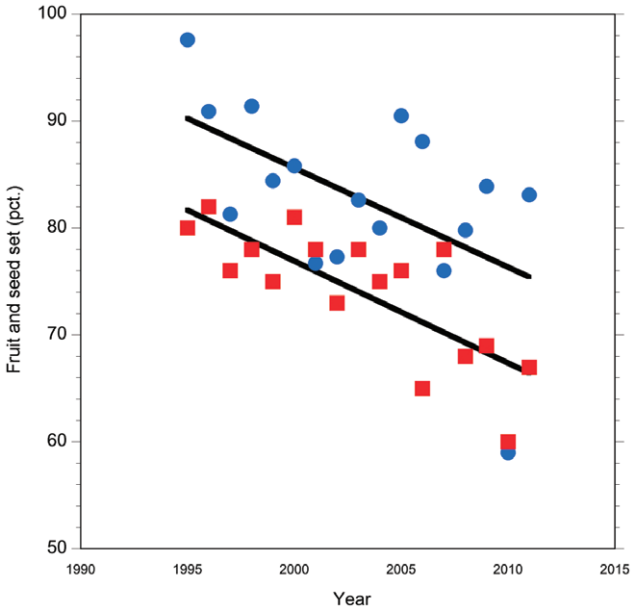

Figure 3. Reproductive success of *Borderea chouardii* over 17 years. Reproductive success was estimated as fruit set of individual plants (blue dots; percentage of fruits to flowers) and seed set (red squares; percentage of seeds to ovules).
doi:10.1371/journal.pone.0044657.g003

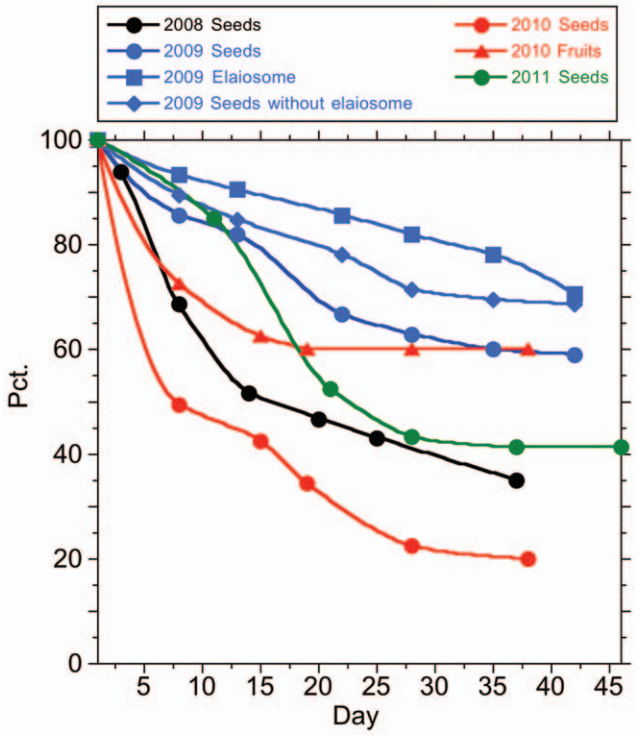

Figure 4. Removal of different kinds of seed items of *Borderea chouardii* from *in situ* cafeteria experiments. Categories are seeds with elaiosome (2008, 2009, 2011), seeds without elaiosome (2009), individual elaiosomes (2009) and entire open fruits containing 6 seeds (2010).
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vials and bringing them into nearby crevices. The other two ants recorded in the monitoring area (*Lasius cinereus, L. grandis*), the same that visited flowers four months earlier, were observed in the cliff side but no interaction with the plant or vials was recorded. Nevertheless, in spring 2011, two seedlings were observed to root in active nests of *Lasius* spp.

In the *in situ*–cafeteria experiment, both seeds and entire fruits were removed from vials (Fig. 1G, 4). Seed removal rate in cafeteria experiments varied between 40–80% of all seeds offered in vials during six weeks (Fig. 4). Seeds with elaiosome were removed more intensively in 2008 than in 2009, but not significantly faster than elaiosomes alone ($\chi = 1.73$, $P = 0.08$) or seeds without elaiosome ($\chi = 1.43$, $P = 0.15$).

Experiment II. The *ex situ*–cafeteria experiment with *Pheidole pallidula* clearly showed that it preferred seeds of *B. chouardii* to those of any of the other three species (*B. chouardii vs. B. pyrenaica*: $\chi = 3.60$, $P = 0.0003$; *vs. Sarcocapnos enneaphylla*: $\chi = 5.77$, $P = 0.0001$; and *vs. Silene acaulis*: $\chi = 6.30$, $P = 0.0001$). However, seeds of both *Borderea* species were preferred to seeds of the other species (Fig. 5).

Experiment III. All 30 seeds of *B. chouardii* placed in front of *P. pallidula* laboratory nests were harvested. Sixty–three percent were predated, while the rest were discarded intact. Both *Lasius grandis* and *L. cinereus* left the elaiosome and the seed coat untouched, i.e. all seeds remained intact. All seeds offered to *L. cinereus* remained outside the nest before and after hibernation. The response of *L. grandis* in young nests before hibernation varied. Before and after hibernation, 1/3 of the nests had seeds inside the nests.

Successful Seed Dispersal and Survival of Seedlings

During 1995–2011, the estimated proportions of *S*–, *A*– and *G*–seedlings were 51%, 39% and 10% respectively ($N = 139$ seedlings). Our *G*-seedlings, however, could contain some *A*-seedlings too because ants can move seeds downwards in the crevice. If dispersal away by ants from the mother plant was random, and we name *a* as total *A* and *g* as total *G*, we would expect for non self-sowed seedlings: (100%–51%) = 49% = *a* + *g*, and for seedlings 10 cm away below a female: 10% = *a* *(45°/360°) + *g* (Fig. 2). Thus our best estimate of the proportion of seedlings dispersed by *g* becomes 4.4% and that of *a* becomes 44.6%.

Survival probability of 1-year old seedlings was 63% after *A* ($N = 43$) and 70% after *S* ($N = 69$) and the difference was non–significant ($\chi = 0.74$, $P = 0.46$).

Discussion

Borderea chouardii is dioecious, which precludes any self–pollination. In addition, we ruled out wind–pollination experimentally, leaving animal pollination as our only remaining option. However, in spite of many hours of observation of flowers, only three species of ants (*Lasius grandis*, *L. cinereus*, and *Camponotus cruentatus*) were observed as visitors attracted by the nectar, besides a few collembolas and parasitic hymenopterans. *Borderea chouardii* does have several characteristics associated with ant pollination, especially easily accessible nectar, low growth form, and small flowers being less attractive to larger insects [2,28]. Thus we conclude that *B. chouardii* is ant–pollinated, but that the visitation rate of ants is as low as *c.* 1 ant/plant/hour. Its reproductive success is high, although we observed a steady decline over the years. The congeneric *Borderea pyrenaica*, also restricted to the Pyrenees, but growing on screes, is ant–pollinated as well and has a similar seed production [6]. Here, an experimental study demonstrated that pollen transported by its ant pollinator

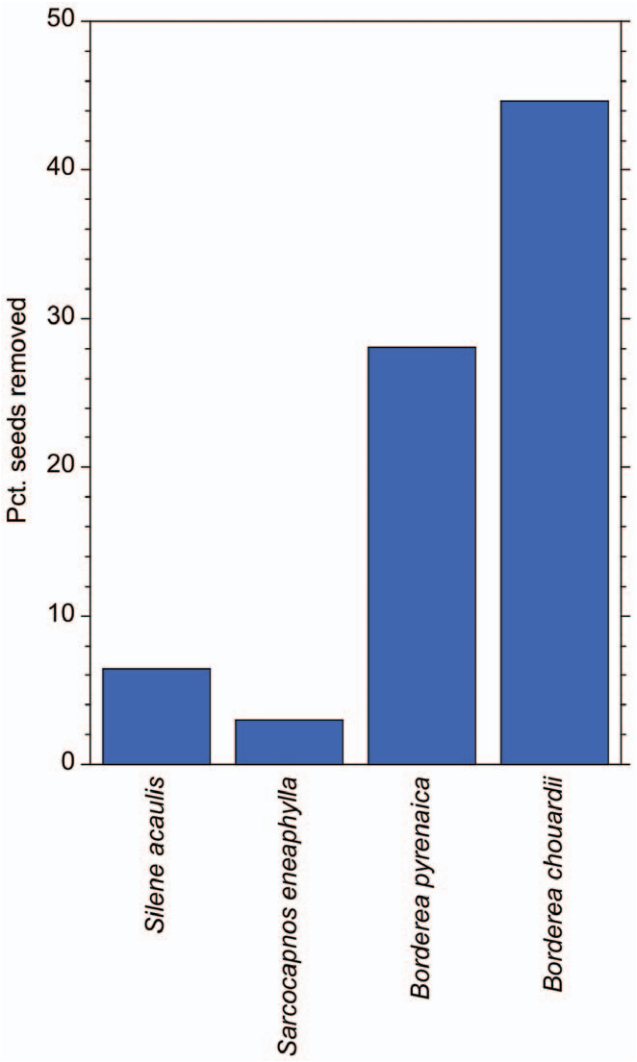

Figure 5. Frequency of seed removal of four plant species in natural nests of the ant *Pheidole pallidula*. Bch = *Borderea chouardii*, Bp = *Borderea pyrenaica*, Se = *Sarcocapnos enneaphylla*, and Sa = *Silene acaulis*.
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Leptothorax tuberum was viable [6]. Thus, ants are successful pollinators of the only two species in this tropical relict, montane, endemic plant genus.

Seeds of *B. chouardii* were dispersed by self-sowing, ants, and/or gravity. Self-sowing was a likely dispersal mode because of the unusual skototropic behavior, i.e. elongation towards nearby dark crevices, of fruiting pedicels [29]. Successful skototropic events, although not frequent (9%), ended up in fruits ripening within crevices, where they dehisced and the seeds were released [Fig. 1E].

A priory, ants were seed–disperser candidates because of the elaiosome of the seeds, which is unique in the family Dioscoreaceae (mostly wind-dispersed), and is, in general, a key adaptation to ant–seed dispersal. Ants were never directly observed gathering the seeds from plants on the cliff, but three species (*Lasius cinereus*, *L. grandis*, *Pheidole pallidula*) were observed on the wall near plants with ripe fruits. The latter species was observed to remove seeds from vials and bringing them into nearby crevices, and some seedlings were found to be rooted in active *Lasius* nests. Skototropism also increased ant's probability to encounter fruits in the cliff, and resulted in an increased and less variable ambient temperature to the fruits due to the higher specific heat capacity of the rock compared to air. This could accelerate ripening, which might become increasingly important with the decline in ant abundance on the rock walls in the early autumn. This late-seasonally ant–seed dispersal of *B. chouardiii* seems to be unique among temperate ant–dispersed plants in general [30], which most often are fruiting in spring or early summer.

Seeds with or without elaiosome were removed from the vials with similar rate (Experiment I). *Pheidole pallidula* showed a preference for the seeds of *Borderea chouardii* compared to seeds with elaiosome of other species, but also harvested seeds of its congener *B. pyrenaica*, without elaiosome but with the same kind of oily coat (Experiment II). *P. pallidula* predated 2/3 of all *B. chouardii* seeds collected and left 1/3 intact (Experiment III). We conclude that *P. pallidula* was a seed disperser of *B. chouardii*, but its price in predated seeds for its mutualistic services was probably high [14,31]. *Lasius* species also harvested the seeds of *B. chouardii*, did not predate them, and their interest in the elaiosome was uncertain (Experiment III). This ant genus is a well–known group of seed dispersers [30,32], and it must disperse seeds of *B. chouardii* because some seedlings have been found to grow in its nests. In contrast to *Pheidole*, *Lasius* ants have never been recorded as seed predators (according to the FORMIS 2009 database) [33].

Finally, gravity was also a likely mode of dispersal because of the vertical habitat. However, it seems to be of minor importance given the low frequency of new recruited seedlings by this dispersal mode. The reason is probably the combination of skototropism and ants, together with the low chance of being retained in the few crevices available when seeds are released.

Our conclusion is that ants serve as both pollinators and seed dispersers of *B. chouardii*. This is one of the very few records of ants as double mutualists. However, the species runs a double jeopardy putting all its stakes on just one kind of mutualist. Only a very long-lived plant can reduce that risk, because longevity confers demographic stability and increases the independence from recruitment [28,29]. In fact, *B. chouardii* probably holds the astonishing world record in individual lifespan among non-clonal plants: >300 years (Fig. 1I). About 1,000 plants may grow on the monitored area, i.e. about 700 males and 300 females. During 17 years of population monitoring, 139 seedlings were recorded. That is a mean of only 8.2 per year or 0.03 per female and year. In spite of this low recruitment, the demographic dynamics of the species is one of the most stable known among herbaceous plants [21].

Rocky habitats are widespread, but the ecology of their inhabitants is poorly known because of obvious accessibility problems. Consequently, they are among the least disturbed places on our planet, and play a major role as natural reserves for many rare and endemic species [18]. Rocky habitats, therefore, are of outstanding value to conservation of biodiversity. However, rock–living plants experience strong selection from especially nutrient deficiency, shortage of recruitment sites and the detrimental consequences of gravity to seed dispersal. Ants can mitigate this by offering mutualism services and nutrient-rich recruitment sites.

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Author Contributions

Conceived and designed the experiments: MBG JMO. Performed the experiments: MBG XE JMO. Analyzed the data: MBG JMO. Contributed reagents/materials/analysis tools: MBG JMO. Wrote the paper: MBG JMO.

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Fear of Predation Slows Plant-Litter Decomposition

Dror Hawlena *et al.*

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most likely caused by the pulsed nature of our sulfide supply. This may have led to short periods of exposure of *Zostera* to toxic sulfide levels.

Coastal ecosystems, and seagrass meadows in particular, are currently declining at an alarming and increasing rate worldwide, leading to loss of biodiversity (*1*). Extensive restoration efforts have had little success so far (<30%), despite their extremely high costs (±$100,000 per hectare) (*23*). Similar to the function of mycorrhizae, pollinators, or seed dispersers in terrestrial systems (*24*–*26*), our findings indicate that restoration efforts should not only focus on environmental stressors such as eutrophication, sediment runoff, or high salinity as a cause of decline but should also consider internal ecological interactions, such as the presence and vigor of symbiotic or mutualistic relations. Breakdown of symbiotic interactions can affect ecosystem functioning, with bleaching events in coral reefs as a clear example (*27*). Similar to the well-known symbiosis between corals and their unicellular algal endosymbionts (*28*), we conclude that symbioses, rather than one defining species, forms the foundation of seagrass ecosystems.

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Supplementary Materials

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Materials and Methods
Supplementary Text
Figs. S1 to S4
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References (*29*–*119*)

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Fear of Predation Slows Plant-Litter Decomposition

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Aboveground consumers are believed to affect ecosystem functioning by regulating the quantity and quality of plant litter entering the soil. We uncovered a pathway whereby terrestrial predators regulate ecosystem processes via indirect control over soil community function. Grasshopper herbivores stressed by spider predators have a higher body carbon-to-nitrogen ratio than do grasshoppers raised without spiders. This change in elemental content does not slow grasshopper decomposition but perturbs belowground community function, decelerating the subsequent decomposition of plant litter. This legacy effect of predation on soil community function appears to be regulated by the amount of herbivore protein entering the soil.

The quantity and quality of detrital inputs to soil regulate the rate at which microbial communities perform ecosystem processes such as decomposition, nitrogen (N) mineralization, and carbon (C) sequestration (*1*, *2*). Because uneaten plant litter makes up the majority of detritus (*3*), it is assumed that these belowground ecosystem processes are only marginally influenced by biomass inputs from higher trophic levels in aboveground food webs, such as herbivores themselves (*4*). We provide evidence here, however, that predators may influence the decomposition of plant litter via a legacy effect of predation risk. Specifically, a physiological stress response to the risk of predation changes the elemental content of herbivore biomass. In turn, the decomposition of these stressed herbivores alters the function of belowground communities, leading to an overall decrease in the decomposition of plant litter.

Our work addresses whether food web structure (especially the existence of predators) influences ecosystem functioning via changes in the nutritional contents of prey (*5*, *6*). The prevailing view is that food web structure does not influence prey body C-to-N (C:N) contents, because to survive and reproduce, prey must maintian relatively constant body C:N ratios (*7*). However, this view assumes that predator effects on prey are entirely consumptive (*5*). Instead the presence of predators generates fear, leading to physiological stress responses in prey, such as elevated metabolism and the synthesis of heat shock proteins (*8*). Together, these stress responses increase basal energy demands (*9*–*12*) that, in nutrient-limited systems, reduce the energy available for the competing demands of production (that is, reproduction and growth) (*13*). Thus, to meet heightened maintenance-energy demands, stressed herbivores divert energy from production, as well as increase their consumption of energy-rich carbohydrates (*12*). Given that the amount of energy used for production correlates positively with N demand, and that herbivores have limited ability to store excess nutrients, stressed herbivores should also excrete more N (*8*, *14*). N excretion is further enhanced because chronically heightened stress hormone levels increase the breakdown of body proteins to produce glucose (*15*). Ultimately, prey stressed by predation risk should increase their body C:N ratio (*8*), and this is observed in field and laboratory experiments (*12*, *16*).

In this study we asked whether predators can regulate plant-litter decomposition through

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indirect influences on the elemental stoichiometry of their prey. Our working hypothesis is that the C:N content (quality) of herbivore biomass entering soils as detritus induces changes in how soil communities process other resource inputs such as plant litter. Such legacy effects of resource quality have been observed in litter-decomposition studies (*17*). Moreover, simple organic compounds may prime soil communities in ways that enhance the decomposition of more-complex organic compounds (*18*).

Using a combination of laboratory and field experiments, our work built on examinations of food web effects on prey physiological stress and elemental stoichiometry in a well-studied grassland ecosystem including the spider predator *Pisaurina mira*, a dominant grasshopper herbivore (*Melanoplus femurrubrum*), and a variety of grasses and forbs (*12*). We reared grasshoppers in the field with the risk of spider predation (stress treatment) and without (control) (*19*). The body C:N content of stressed grasshoppers (mean ± SE, 4.00 ± 0.03; N = 11.62 ± 0.12%) was significantly higher (Wilcoxon signed-rank test $z = -2.023$; $P < 0.05$) than that of nonstressed grasshoppers (3.85 ± 0.04; N = 12.11 ± 0.16%). We then used carcasses of these grasshoppers in laboratory microcosm experiments to test whether the difference in body elemental composition altered the decomposition of the grasshoppers and subsequent plant litter inputs. We added a small amount (3.5 mg) of either stressed or nonstressed grasshopper biomass to microcosms containing soil collected from our grassland ecosystem. Carbon mineralization rates of grasshopper biomass were monitored until rates did not differ from reference microcosms containing only soil. At this time (42 days), there was little difference in cumulative C mineralization (that is, the total amount of C respired as CO_2 across the entire incubation) between stressed and stress-free grasshopper treatments (Fig. 1A). This was not unexpected, given that both stressed and stress-free grasshoppers represent high-quality resources to belowground communities. More interesting was how small elemental changes in these inputs might affect the subsequent functioning of soil communities, especially the decomposition of lower-quality substrates such as plant litter (*18*)

To test the functional implications of slight nutrient differences in high-quality resource inputs, we added grass litter (500 mg) to the microcosm soils previously amended with stressed or stress-free grasshopper carcasses and then measured C mineralization. After 118 days, mineralization of the grass litter in soils previously amended with stressed grasshoppers was 62% lower ($F_{1,4} = 13.9$, $P < 0.05$) than that of the same litter in soils amended with nonstressed grasshoppers (Fig. 1B). Thus, the small input of herbivore biomass (~140 times less than the added litter mass), coupled with a 4% difference in the C:N ratio between stressed and nonstressed grasshopper carcasses, caused a threefold difference in the mineralization of plant-litter inputs. These results suggest a causative link between predation-induced changes in prey body chemistry and altered soil community function. The most plausible explanation for why such a small shift in prey C:N ratio might

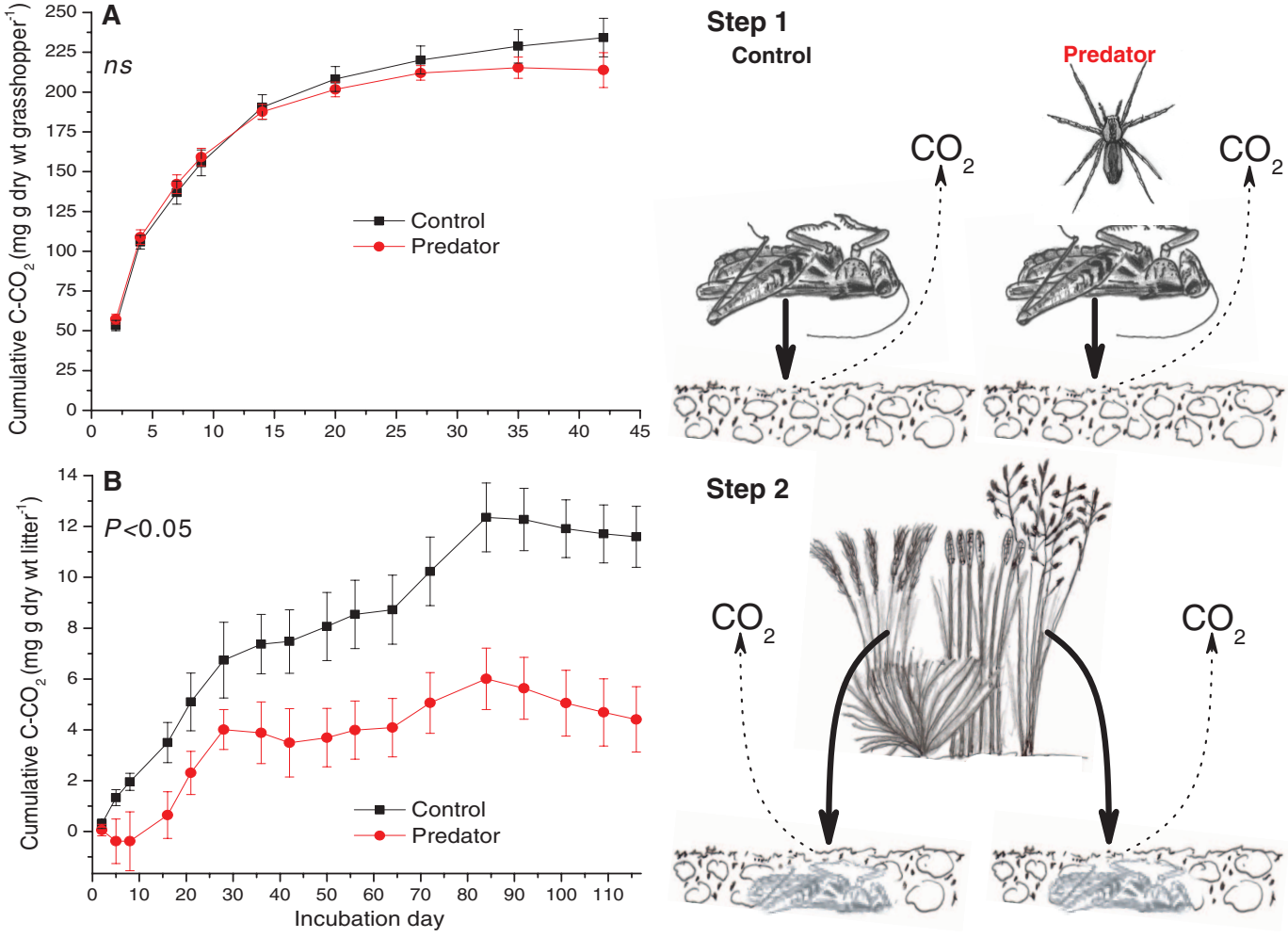

Fig. 1. Cumulative C mineralization (mean ± 1 SE, n = 5 microcosms) during decomposition of (**A**) nonstressed grasshoppers versus those stressed by predators (step 1); and (**B**) grass litter added to the same microcosms (step 2) after the completion of the grasshopper decomposition experiment shown in step 1. Although control and stressed grasshoppers were mineralized at similar rates ($P > 0.05$), the addition of grasshopper carcasses reared with disarmed predators led to subsequent reductions in plant-litter decomposition rates ($P < 0.05$). Rates are differences from microcosms not amended with grasshoppers, so cumulative values can be negative.

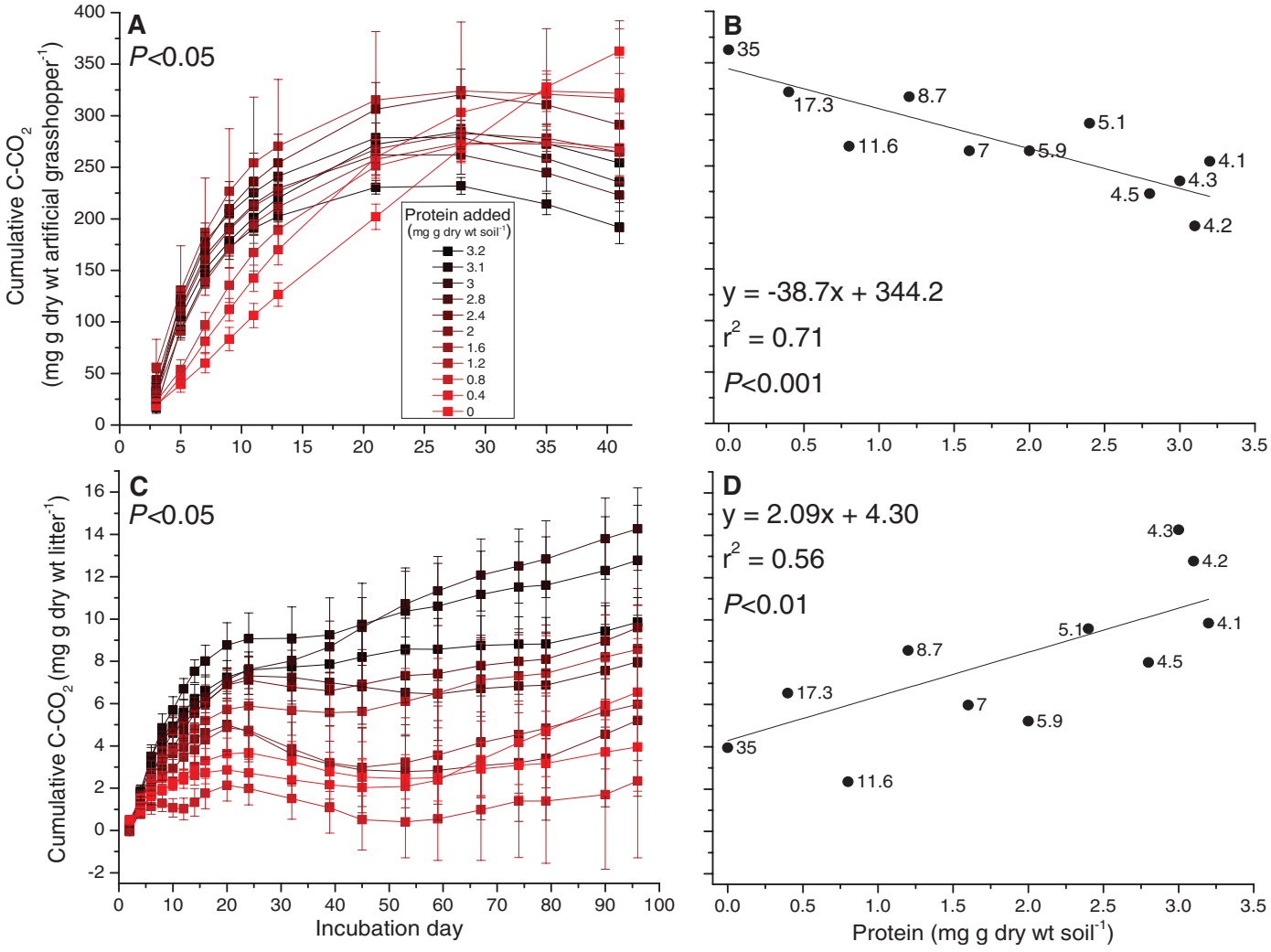

Fig. 2. (**A**) Cumulative C mineralization (mean ± 1 SE, n = 6 microcosms) during decomposition of artificial grasshoppers with varying C:N ratios and protein contents and (**B**) the relationship between the amount of protein added and the cumulative mineralization associated with these artificial grasshoppers. (**C**) The cumulative C mineralization (mean ± 1 SE) of grass litter added to the same microcosms after completion of artificial grasshopper decomposition and (**D**) the relationship between the amount of protein added and subsequent grass-litter mineralization. Rates are differences from microcosms not amended with artificial grasshoppers, so cumulative values can be negative [as in (C)]. The C:N ratios associated with the different artificial grasshoppers are shown next to their respective points.

translate to such a large shift in litter mineralization rates is that small increases in N availability can prime the activities of decomposer microorganisms and hence accelerate litter mineralization (*20*). We next examined whether the observed effects on litter mineralization were probably attributable to the variation in the C:N content of prey tissue being linked to N availability and hence priming of microbial activity.

To test whether prey body C:N content influenced plant-litter decomposition, we again used a laboratory microcosm approach, but this time manipulated C:N ratios by creating "artificial grasshoppers" (*19*). To simulate grasshopper tissue, we used 4-mg organic matter mixtures of 20% chitin and varying proportions of carbohydrates (0 to 80%) and proteins (0 to 80%), generating C:N ratios that spanned more than the observed variation. Carbon mineralization of the artificial grasshoppers was measured (for 40 days)

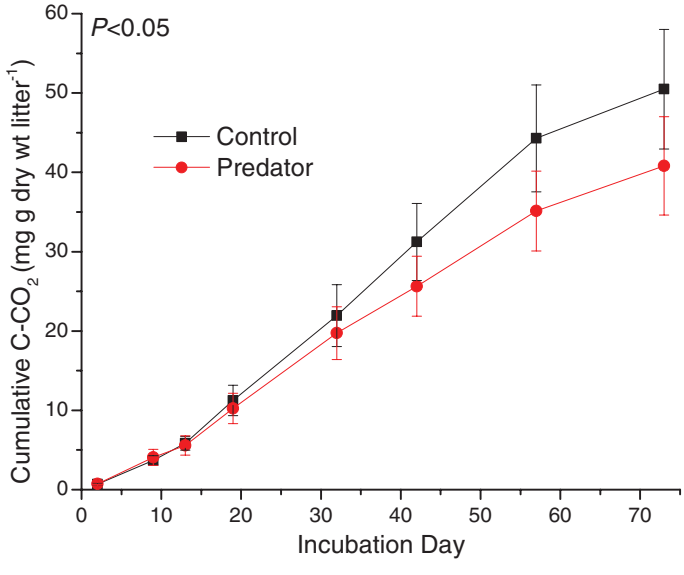

Fig. 3. Cumulative C mineralization (mean ± 1 SE, n = 7 field plots) of ^{13}C-labeled grass litter decomposed in blocked field plots, first amended with control grasshoppers or those stressed by predator presence.

until it approximated rates in reference microcosms containing only soil. Cumulative C mineralization varied by up to twofold across the artificial grasshopper treatments ($F_{10, 55} = 2.15$, $P < 0.05$; Fig. 2A). Notable, given the broad range in C:N across treatments, is the positive relationship between the C:N of artificial grasshoppers and the cumulative C-mineralization, as well as the negative relationship between mineralization and amount of protein (Fig. 2B). These observations are most likely explained by higher growth efficiencies of soil organisms. This might be expected because organisms consuming resource inputs with lower C:N ratios and consequently higher protein levels will favor production over waste respiration and hence reduce total C mineralization (*20*).

The availability of protein N is essential both for microbial production and for facilitating the decomposition of organic matter, because it is used to produce extracellular enzymes that catalyze the degradation of complex C compounds (*20*–*22*). It then follows that the carcasses of stressed grasshoppers, which have higher biomass C:N, probably because of lower body protein levels (*23*), provide less available N and thus should retard plant-litter decomposition. Further support for this interpretation came when we added grass litter (500 mg) to the microcosms previously amended with artificial grasshoppers. Across 96 days, the mineralization rates of grass litter diverged by as much as sixfold ($F_{10, 55} = 2.34$, $P < 0.05$; Fig. 2C), despite the only twofold difference in the cumulative mineralization of artificial grasshoppers. These results mirror, qualitatively, our first experiment with real grasshoppers, in which lower available N led to reductions in the decomposition of plant litter (Fig. 2, C and D). These results also show that varying C:N ratios only partially explain altered plant-litter mineralization. Specifically, the protein content of artificial grasshoppers, for which the C:N ratio is a common but indirect index (*23*), has over twice the power [coefficient of determination-explained variance (R^2) = 0.56, $F_{1,10} = 13.5$, $P < 0.01$; Fig. 2D] of the C:N ratio ($R^2 = 0.23$, $F_{1,10} = 4.0$, $P = 0.08$) to explain plant-litter decomposition rates. This is probably because the C:N ratio is influenced by both labile and recalcitrant N-bearing compounds. Consequently, a small difference in the C:N ratio may reflect much larger variation in protein N content. Together, the laboratory experiments reveal a potentially important general mechanism (*8*) by which predators regulate soil ecosystem processes through stress-induced changes to herbivore nutrient content. It remained uncertain whether this mechanism explains variation in belowground community function in nature.

To test for predator-induced regulation of decomposition variation in mineralization rates under natural conditions, we added intact carcasses of grasshoppers reared either with predation risk (stress treatment) or without (control) to field plots. After 40 days, we added ^{13}C-labeled grass litter (550 g m^{-2}) to the same plots and measured ^{13}C mineralization in situ, using cavity ring-down spectroscopy, a highly sensitive form of laser absorption spectrometry that quantifies the stable isotope composition of C in CO_2 (*19*). Using ^{13}C labeling meant that we could separate the contribution of mineralization of the added litter from total soil respiration. After 73 days, mineralization of the grass litter, in plots amended with stressed grasshoppers, was 19% lower ($F_{1,6} = 9.06$, $P < 0.05$) than in plots that received stress-free grasshoppers (Fig. 3). This mechanism is not mutually exclusive of other mechanisms that regulate soil communities (*4*, *21*, *24*). Nonetheless, in our experiments, the effect of predation on litter decomposition had a measureable impact through changes in the nutrient content of herbivore carcasses. These results highlight the potential for this mechanism to influence decay rates of organic matter inputs and hence ecosystem C and N cycling.

A key remaining question is whether predator-induced changes persist when multiple aboveground and belowground pathways act simultaneously. We examined this question by rearing grasshoppers in field plots where predation risk was absent (no spiders) or present (spiders with glued mouthparts) (*19*). Toward the end of the growing season and 1 month after the grasshopper adults had reproduced, died, and been allowed to decompose, we transferred surface soil from the field plots to laboratory microcosms. We found no difference in soil pH between risk and risk-free field mesocosms ($F_{1,6} = 0.761$, $P = 0.417$). To test for legacy effects of fear, we then added grass litter to the microcosms and measured C mineralization for 93 days. Cumulative mineralization of grass litter, amended to soil communities developed under the predator treatment, was ~200% lower than litter mineralization from communities developed without spider predators ($F_{1,6} = 6.23$, $P < 0.05$; Fig. 4). Collectively, our experiments suggest that cascading effects of predation risk are measurable on litter decomposition in the field and laboratory and occur through predator-induced changes in prey chemical composition.

Traditional concepts of trophic pyramids in ecosystems highlight the idea that inputs of plant-derived materials to soils are more important for regulating belowground processes than are inputs from other trophic levels, because plant inputs are dominant (*4*). Accordingly, predators are presumed to regulate ecosystem processes mainly by altering the quality and quantity of plant-derived materials entering belowground systems, through the control of herbivore density (that is, through trophic cascades) and/or by altering herbivore foraging behavior (*4*, *5*). Our work instead suggests that predators can regulate ecosystem processes more directly through stress-induced changes in the chemical composition of prey body tissue.We find that small additions of high-quality herbivore biomass influence the decomposition of much larger inputs of recalcitrant plant litter, with effects lasting for at least the duration of a normal growing season (80 to 110 days). Indeed, we show that predator-induced changes in the nutritional composition of herbivore biomass dramatically slow the decomposition of plant litter through legacy effects on soil communities. Our work suggests that the mechanism governing these effects is the amount of animal protein that enters the soil. Our work adds to the body of recent work (*5*, *25*) showing that predators exert top-down control, through multiple mechanisms, on ecosystem processes. Evaluating the importance of these newly identified roles of predators in ecosystems is made all the more urgent because we are losing them from ecosystems at disproportionately higher rates than other species (*25*, *26*).

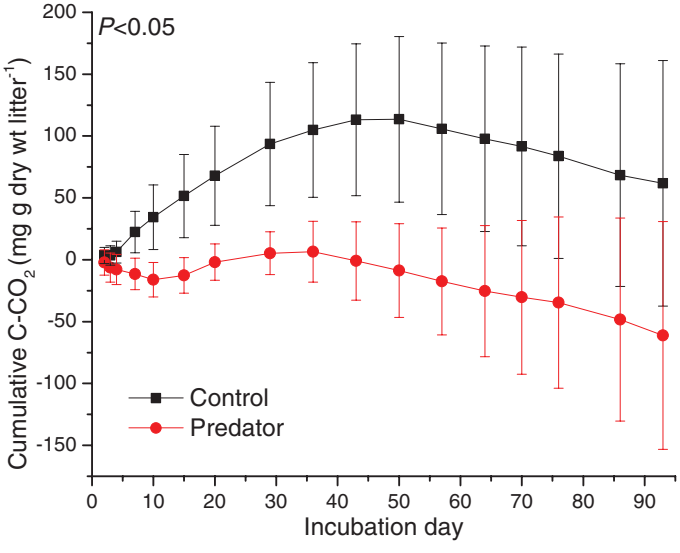

Fig. 4. Cumulative C mineralization (mean ± 1 SE, $n = 7$ microcosms) of grass litter on soil collected from blocked field plots with or without predation risk. Rates are differences from reference soils, so cumulative values can be negative.

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Supplementary Materials

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Continental-Scale Effects of Nutrient Pollution on Stream Ecosystem Functioning

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Excessive nutrient loading is a major threat to aquatic ecosystems worldwide that leads to profound changes in aquatic biodiversity and biogeochemical processes. Systematic quantitative assessment of functional ecosystem measures for river networks is, however, lacking, especially at continental scales. Here, we narrow this gap by means of a pan-European field experiment on a fundamental ecosystem process—leaf-litter breakdown—in 100 streams across a greater than 1000-fold nutrient gradient. Dramatically slowed breakdown at both extremes of the gradient indicated strong nutrient limitation in unaffected systems, potential for strong stimulation in moderately altered systems, and inhibition in highly polluted streams. This large-scale response pattern emphasizes the need to complement established structural approaches (such as water chemistry, hydrogeomorphology, and biological diversity metrics) with functional measures (such as litter-breakdown rate, whole-system metabolism, and nutrient spiraling) for assessing ecosystem health.

Nutrient enrichment from organic inputs and agricultural run-off is placing the world's vulnerable fresh waters in a precarious position (*1*–*4*). Far-reaching environmental legislation has been introduced to redress human impacts on aquatic communities (*5*, *6*), yet the consequences of nutrient loading for stream ecosystem functioning remain poorly understood (*4*, *7*, *8*). This is worrying because key ecosystem services (such as maintenance of viable fisheries as a provisioning service, and organic matter decomposition as a supporting service) ultimately depend on ecosystem processes, such as leaf-litter breakdown and other processes involved in nutrient cycling (*3*, *9*).

Many aquatic ecosystems are supported by plant litter inputs (*10*–*12*). This includes streams, where terrestrial leaf breakdown—which is driven by resource quality; the abundance, diversity, and activity of consumers; and environmental factors—is a key ecosystem process (*10*, *13*, *14*). Moderate nutrient enrichment of streams can accelerate breakdown by stimulating microbial conditioning and invertebrate consumption (*15*, *16*). However, a wide range of responses along nutrient gradients has been reported in field studies, suggesting environmental drivers beyond elevated nutrient supply. For instance, wastewater discharge can induce anoxia, mobilize heavy metals, and physically smother benthic organisms (*17*, *18*). Litter breakdown by invertebrates (*19*) appears especially sensitive to nutrient pollution relative to that mediated by microbes (*20*) and, because invertebrates often attain their highest densities in moderately enriched streams, a hump-shaped breakdown rate response might be expected along long nutrient gradients (*5*).

We hypothesized that breakdown rates are constrained by microbial nutrient limitation at the low end of nutrient pollution gradients and by the effects of environmental degradation on invertebrates at the high end. Most studies, however, have been unable to detect this pattern because they have been conducted over relatively short nutrient gradients and small spatial scales (*5*, *7*). Here, we report a field experiment in 100 European streams spanning 1000-fold differences in nutrient concentrations, as proxy measures of nutrient loading by direct and indirect inputs (*21*). The validity of this approach is highlighted by the positive relationship between biochemical oxygen demand (BOD_5) and nutrient concentrations in more than 8000 European streams, and the comparable frequency distributions of nutrient concentrations between these and our sites (fig.

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Brain imaging reveals neuronal circuitry underlying the crow's perception of human faces

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Crows pay close attention to people and can remember specific faces for several years after a single encounter. In mammals, including humans, faces are evaluated by an integrated neural system involving the sensory cortex, limbic system, and striatum. Here we test the hypothesis that birds use a similar system by providing an imaging analysis of an awake, wild animal's brain as it performs an adaptive, complex cognitive task. We show that in vivo imaging of crow brain activity during exposure to familiar human faces previously associated with either capture (threatening) or caretaking (caring) activated several brain regions that allow birds to discriminate, associate, and remember visual stimuli, including the rostral hyperpallium, nidopallium, mesopallium, and lateral striatum. Perception of threatening faces activated circuitry including amygdalar, thalamic, and brainstem regions, known in humans and other vertebrates to be related to emotion, motivation, and conditioned fear learning. In contrast, perception of caring faces activated motivation and striatal regions. In our experiments and in nature, when perceiving a threatening face, crows froze and fixed their gaze (decreased blink rate), which was associated with activation of brain regions known in birds to regulate perception, attention, fear, and escape behavior. These findings indicate that, similar to humans, crows use sophisticated visual sensory systems to recognize faces and modulate behavioral responses by integrating visual information with expectation and emotion. Our approach has wide applicability and potential to improve our understanding of the neural basis for animal behavior.

American crow | cognition | facial recognition | [F-18]fluorodeoxyglucose–PET imaging | learned fear

A variety of species are able to discriminate between human faces (1–3), and this ability appears to be linked to neural integration of perception, emotion, and memory. Brain imaging studies have revealed that humans use a core recognition system in their sensory cortex (the posterior superior temporal sulcus, the inferior occipital gyrus, and the fusiform gyrus) networked with two extended systems that convey the historical (anterior paracingulate, posterior superior temporal sulcus/temporoparietal junction, anterior temporal cortex, precuneus, and posterior cingulate) and emotional (amygdala, insula, and striatum) significance of the person (4). This network of brain regions that perceive and analyze faces is informed by ventral and dorsal visual pathways—the ventral enabling fine discrimination and the dorsal providing rapid, but coarse, emotional assessment (3). Brain mapping investigations on other species capable of human recognition are extremely limited; however, electrophysiological recordings in the visual cortex of domestic sheep and nonhuman primates have indicated the presence of neurons that respond to human facial information (5).

We demonstrated previously that free-ranging American crows (*Corvus brachyrhynchos*) discriminate among humans based on facial characteristics, but we could only speculate on the neural basis for this behavior (1, 6). Because birds and mammals share some common sensory and motor circuits (7), we hypothesized that recognition of humans by crows might involve a distributed set of interactive brain regions. Here we use a neuroimaging approach to test this hypothesis and investigate the underlying neuronal circuitry activated in response to the sight of familiar people whom we expect crows to recognize as either threatening or not threatening. To accomplish this goal, 12 adult male crows were captured by investigators wearing identical masks, a process which we had previously demonstrated was sufficient for crows to learn the masks as a "threatening" face (1). Over their 4-wk captivity, crows were fed by caregivers wearing an alternative, "caring," mask (Fig. 1). We used positron emission tomography (PET) combined with administration of [F-18]fluorodeoxyglucose (FDG) to assess the brain activity of wild crows responding adaptively to these faces. During an uptake phase when FDG accumulates in the brain proportional to regional brain activity, we kept the awake crow in a controlled physiologic condition and showed it one of the following: (*i*) a person wearing the mask that captured it, (*ii*) a person wearing the mask that fed it, or (*iii*) an empty room. Once FDG was predominantly fixed in the brain, the subject could be imaged under anesthesia. The resultant images showed brain activity during the uptake phase. Although previously used for human brain mapping research (8), our experiment adapts this technology to map the response of a bird's brain to a natural, visual, cognitive task and allows us to image a nonhuman animal responding to a human face (9, 10).

Results and Discussion

The visual system of birds and primates is supported by perhaps the most advanced and sophisticated neural sensory system known (11). Much of this complexity is evident in the whole-brain responses of crows in our visual discrimination experiments. The pattern of FDG uptake during visual stimulation revealed activation of the crows' tectofugal visual pathway and a diversity of other forebrain regions (Fig. 2 and Movie S1). Our activation paradigm concentrated neural activity on the central fovea of each retina, stimulating a strong response by the nucleus rotundus of the thalamus and especially its target in the forebrain, the entopallium (Fig. 2). In lateral-eyed birds, such as the crow, this visual network resolves distant, complex, and novel objects, and it is important to visual discrimination tasks; pattern recognition; concept formation that enables categories and individuals to be recognized; and depth perception (12, 13)—all tasks relevant to crows in our experiments.

Our results suggest that American crows recognize familiar human faces by evaluating visual sensory information in the context of learned associations. The sight of a familiar human, either threatening or caring, consistently activated the rostral forebrain, including the hyperpallium and a large region in the nidopallium/mesopallium (Fig. 3). Differential activation of the hyperpallium suggests that, in addition to the use of the tectofugal visual

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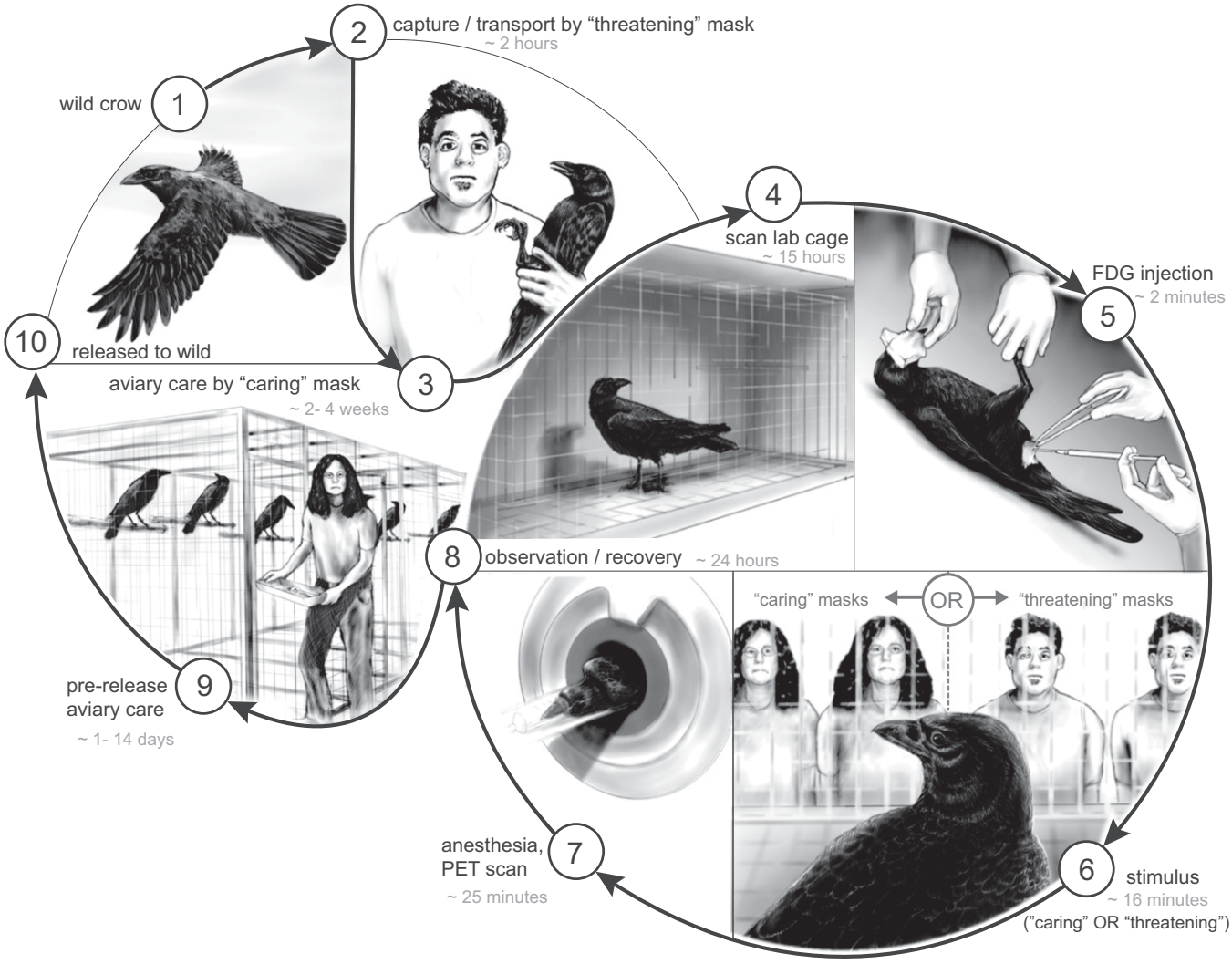

Fig. 1. Experimental protocol. Different rubber masks, molded from actual people, were used to create threatening and caring faces that single crows responded to during stimulation. During i.p. injection and induction, crow's faces were covered to prevent them from glimpsing humans.

pathway, crows used their thalamofugal pathway (directly linking thalamus and hyperpallium) to perceive humans. Activation of the rostral nidopallium, which has strong connections with the entopallium and mesopallium (14) and access to sensory information from both the tectofugal and thalamofugal visual pathways (15), appears important to the recognition of human faces.

Crows quickly associated negative (capture) and positive (provision of food) experiences with a human face. In our stimulation protocol, familiar humans were seated and did not behave as expected, neither threatening nor providing care to crows during stimulation. This surprise, or prediction error, may account for the activation of the crows' lateral striatum (Fig. 3), as it does in a variety of animals, including humans (16). The activated rostral mesopallium, an associative forebrain area that participates in rapid, multimodal learning and is enlarged in crows (17), may be especially important to the association of human faces with reward and punishment. Caudal regions of the nidopallium, mesopallium, and hippocampus—which are important to the recognition of biologically significant conspecifics (18) and executive function (19)—were not consistently activated by the sight of a person.

Crows activated different regions when viewing a familiar threatening vs. a familiar caring person. Upon seeing the face of their captor, crows activated their nidopallium/mesopallium, arcopallium including the region containing the nucleus taeniae of the amygdala (TnA), and nuclei in the dorsal thalamus and brainstem (Fig. 4 *A*–*C*). This response is commonly elicited by the emotion of fear (20, 21). Definitive identification of nuclei in the thalamus and brainstem was not possible given our resolution and the many small, densely packed nuclei, but likely candidates include those involved in visual pathways (nucleus dorsolateralis posterior thalami and nucleus isthmo-opticus) (22), learning pathways (substantia grisea centralis and locus ceruleus) (23), and emotional motor pathways that control vocal and postural responses to predators (area surrounding tractus occipitomesencephalicus, nucleus reticularis, and trigeminal nucleus) (21, 22). The neural response of crows viewing a human who cared for them daily, albeit in captivity, was distinct from the response of birds to a threatening person. The hyperpallium, mesopallium, preoptic area, and medial striatum were strongly activated (Fig. 4 *A* and *C*). These areas are known to be important to associative learning, motivation, and autonomic functions including hunger in vertebrates (17, 24), which suggests that crows perceived the association established between their caretakers and food. In crows, as in other animals including humans, it appears that both the striatum and the amygdala are critical to associative learning, with the former apparently broadly attuned to prediction errors and the latter often attuned to the reliability of threatening cues (16, 20).

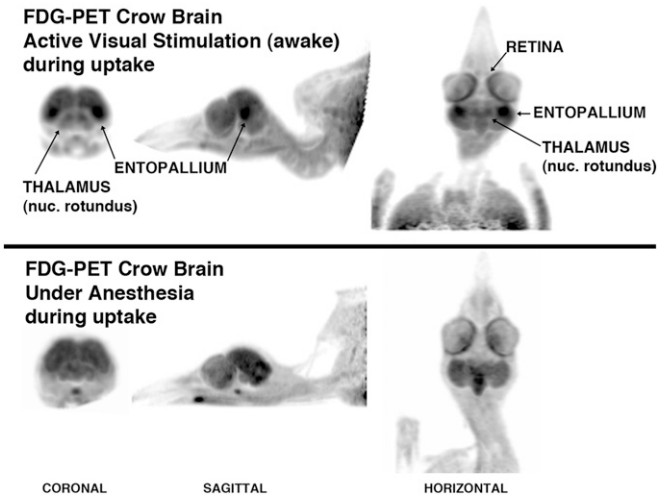

Fig. 2. Exemplar of FDG uptake by a crow. (*Upper*) In this nonquantitative depiction, the FDG-PET brain image has been contrast-enhanced to highlight the fact that the visual network is activated during stimulation following the injection of FDG. (*Lower*) A similar regional distribution of tracer was not observed in the crow brain that was under anesthesia during the uptake period.

The neural responses of crows to threatening and caring faces differed in hemispheric bias, or lateralization, as hypothesized for humans. In support of the valence theory of emotional processing (5), crow responses to the caring (positive) face were predominantly left-hemisphere biased, whereas responses to the threatening face were predominantly right-hemisphere biased. These biases were strongest in the limbic and subpallial structures (threatening, amygdalar regions; caring, preoptic area and striatum; Fig. 4 *A*–*C*). Lateralization was less consistently right-hemisphere biased in the upper pallium (mesopallium, nidopallium, and hyperpallium) to all familiar faces (Figs. 3*A* and 4*A*), perhaps reflecting specialization of the right hemisphere for recognition of familiar social companions (25)—or, alternatively, the tendency of subjects to perch on the right side of the chamber and view the stimulus with their left eye.

Under both experimental conditions and in the field, crows responded to perception of a threatening face with a fixed gaze (quantified as decreased blink rate). In nature, crows blinked less when they looked at threatening people than when they fed in conspecific flocks (Fig. 5*B*). During our experiments, crows froze, stared at the person, and flashed their conspicuous white nictitating membrane on average 29 times per min (SE = 4.4) upon seeing the threatening face. In contrast, when viewing the caring person, crows stared, blinked 41 times per min (SE = 3.4), and often swallowed (caring, two of four birds; threatening, one of five birds) and even defecated (caring, three of four birds; threatening, two of five birds). Reduced blinking at the sight of the threatening person relative to the caring person was as expected from field observations [Mann–Whitney $U = 2.0$; $P = 0.03$ (one-tailed)], but there was substantial variation.

Individual variation in blink rate was associated with distinct brain activity in the crows that viewed human faces. Increased blinking was correlated with increased activity in the hyperpallium and the lateral striatum near the nidopallium (Fig. 5 *A* and *C*). This finding is consistent with processing of visual information by both tectofugal and thalamofugal pathways and integration with expectation from learned associations (15). Reduction in blinking was correlated with increased activity in the brainstem (Fig. 5 *A* and *D*). Blinking and associated neural activity varied continuously among individual crows, suggesting that the birds varied in their perception of, or reaction to, risk and rewards associated with human faces.

The greater overall area of activation, including subthreshold pixels in the nidopallium and mesopallium in the group viewing the threatening vs. that viewing the caring face (Fig. 4), may represent heightened arousal and greater involvement of the crow forebrain to resolve negative vs. positive stimuli. Greater consistency in neural responses to the threatening face suggests differential arousal or attention, but the equal number of significant activation peaks between treatments and the similar demeanor of all crows during testing suggest that crows actually used a larger forebrain area to resolve threatening faces compared with caring faces. During stimulation, crows oriented toward the stimulus and did not fly, vocalize, or flick their wings or tails as is typical in agitated birds. This subdued response across treatments was likely due to the confining nature of the small cage we used. All but one bird moved occasionally during the stimulation protocol, either jumping between the cage floor and perch ($n = 5$) or shifting its head from side to side ($n = 7$). Most of these birds moved only once. However, a single bird in each treatment frequently moved between perch and floor (threatening, 19 movements; caring, 6 movements), and five birds shifted their heads frequently (threatening: mean = 55.5 shifts during stimulation, SD = 43.1, $n = 2$; caring: mean = 71.3, SD = 31.7, $n = 3$).

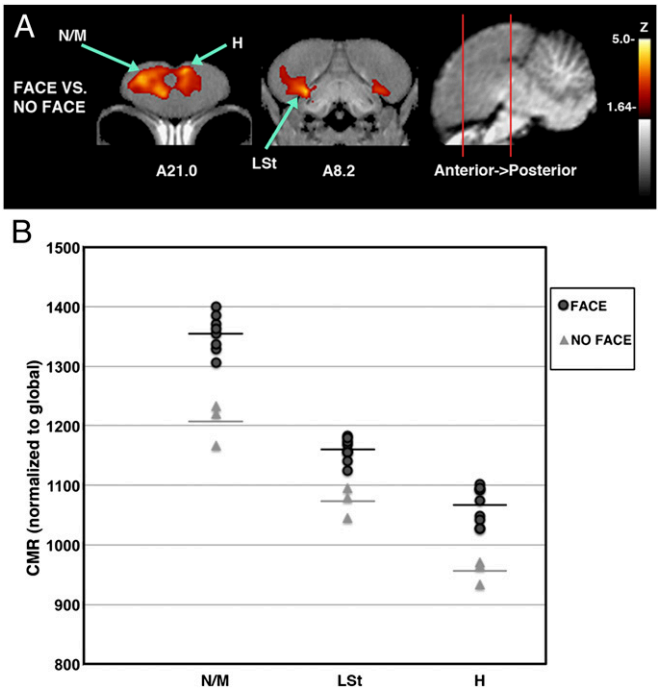

Fig. 3. Differences in brain activation patterns of crows shown familiar human faces vs. no human face. (*A*) The activation pattern of crows viewing a familiar face (either threatening or caring; $n = 9$) compared with a group shown an empty room ($n = 3$) indicated as voxel-wise subtractions converted to group-wise *z*-scores that have been superimposed onto the MRI template for better anatomical localization. Coronal slices (from anterior to posterior; coordinates refer to Japanese jungle crow atlas; ref. 31) illustrate peak activations (voxels with $Z > 1.64$ are colored; those with $Z > 3.8$ are considered significant with associated structures as indicated). (*B*) Individual values for normalized (global) uptake in each structure that met the threshold for statistical significance on *z*-score voxel-wise mapping. Horizontal lines indicate group mean. *Z* values indicated are from peaks in voxel-wise mapping, and *P* values were derived from one-tailed *t* tests of volumes of interest (VOIs) centered on peak activation coordinates. Activated structures: N/M: nidopallium/mesopallium, 12.2% increased, $Z = 4.25$, $P = 0.0000142$; LSt: lateral striatum, 8.1% increased, $Z = 3.99$, $P = 0.000044$; H: hyperpallium, 11.6% increased, $Z = 3.80$, $P = 0.000091$.

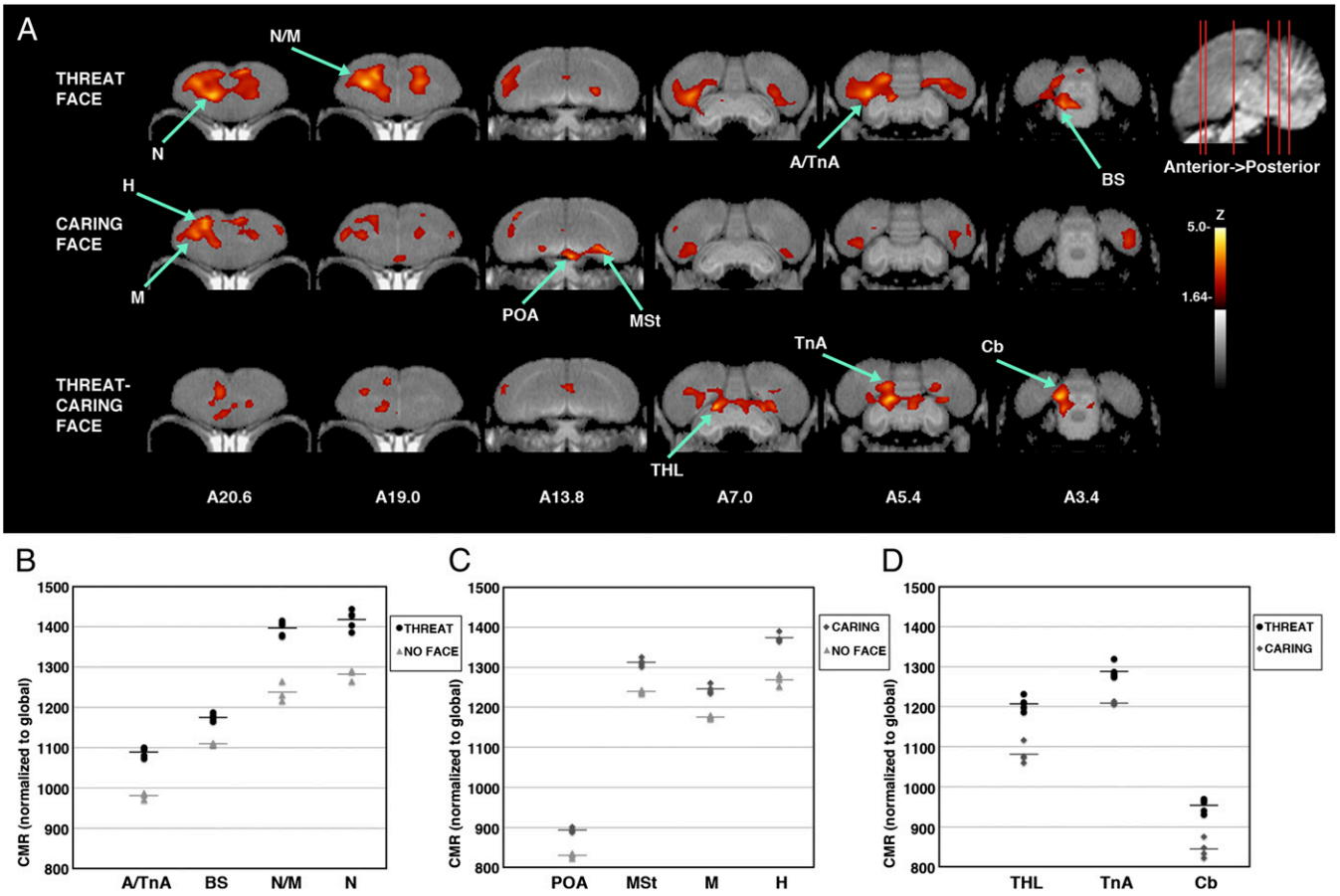

Fig. 4. Brain activation patterns from crows shown human faces that have previously threatened or cared for them. (*A*) As in Fig. 3, voxel-wise subtractions converted to *z*-score maps are superimposed to a structural MRI template of the crow brain for better anatomical localization. (*Top*) The activation pattern of crows viewing a threatening face ($n = 5$) compared with a group shown an empty room ($n = 3$). (*Middle*) The activation pattern of crows shown a caring face ($n = 4$) compared with the empty room group. (*Bottom*) Voxel-wise direct comparison of the group shown a threatening face with those shown the caring face (the areas activated by threatening and not caring faces are indicated). Coronal slices (from anterior to posterior; coordinates refer to Japanese jungle crow atlas; ref. 31) illustrate peak activations in one or more group subtractions (voxels with $Z > 1.64$ are colored; those with $Z > 3.8$ are considered significant with associated structures as indicated). (*B–D*) Individual values for normalized (global) uptake in each structure that met the threshold for statistical significance on *z*-score voxel-wise mapping. Horizontal lines indicate group mean. *Z* values indicated are from peaks in voxel-wise mapping, and *P* values were derived from one-tailed *t* test of VOIs centered on peak activation coordinates. (*B*) Activated structures for threatening face vs. empty room. A/TnA: arcopallium/nucleus taeniae of the amygdala, 11% increased, $Z = 4.42$, $P = 0.00000425$; BS: brainstem nuclei surrounding tractus occipitomesencephalicus including nucleus isthmo-opticus, locus coeruleus, and substantia grisea centralis, 5.9% increased, $Z = 4.26$, $P = 0.0000084$; N/M: nidopallium/mesopallium, 12.9% increased, $Z = 4.14$, $P = 0.000020$; N: nidopallium, 10.6% increased, $Z = 3.93$, $P = 0.000052$. (*C*) Activated structures for caring face vs. empty room. POA: preoptic area, 7.6% increased, $Z = 3.99$, $P = 0.000011$; MSt: medial striatum, 5.9% increased $Z = 3.94$, $P = 0.000052$; M: mesopallium, 5.9% increased, $Z = 3.87$, $P = 0.000091$; H: hyperpallium, 8.3% increased, $Z = 3.81$, $P = 0.000055$. (*D*) Activated structures for threatening face vs. caring face. THL: dorsal thalamus including dorsolateralis posterior thalami, 11.6% increased, $Z = 4.18$, $P = 0.000019$; TnA: nucleus taeniae of the amygdala, 6.5% increased, $Z = 4.02$, $P = 0.000033$; Cb: cerebellum, 12.9% increased, $Z = 3.99$, $P = 0.000043$.

It is unlikely that differences in brain activity were due to extraneous factors. We eliminated variation in environmental factors known to affect brain activity by holding lighting, noise, time of day, room composition, position of observers, handling, and housing before and after treatment constant across trials. The blinking, swallowing, and defecating behavior of crows discussed above suggested that they perceived the difference in stimuli and that they were attentive to both threatening and caring faces. Documenting the neural responses of birds to a variety of threats and rewards could resolve the relative influence of the stimulus on the extent of forebrain activity.

Our results demonstrate how crows use a diversity of regions from the brainstem to the forebrain to distinguish and adjust their response to individual human faces. This finding is consistent with established and emerging views of how the subpallial limbic network interacts with the integrative forebrain to shape memory-based social behavior in vertebrates (17, 18, 26), including humans (27, 28). The use of cortical sensory processing, the striatum, and the limbic system suggests strong analogy and possible homology between avian and mammalian facial recognition and associative learning systems. Further studies are needed to determine whether the forebrain regions used by crows in our study are functionally analogous to facial recognition regions in humans and other mammals.

Our approach that partners neuroscientists with ecologists could be used to better understand the neural bases of cognition in widely diverse animals (29). Current knowledge comes primarily from a few, well-studied, often domesticated species. Neuroimaging of wild animals to assess whole brain activity during complex behaviors, although presently limited to activities that can be elicited in a temporary captive setting, add substantially to traditional lesion, stimulation, and tracing studies (9). In vivo imaging and voxel-wise analyses of brain responses can be repeated longitudinally in the same animals, and when experiments

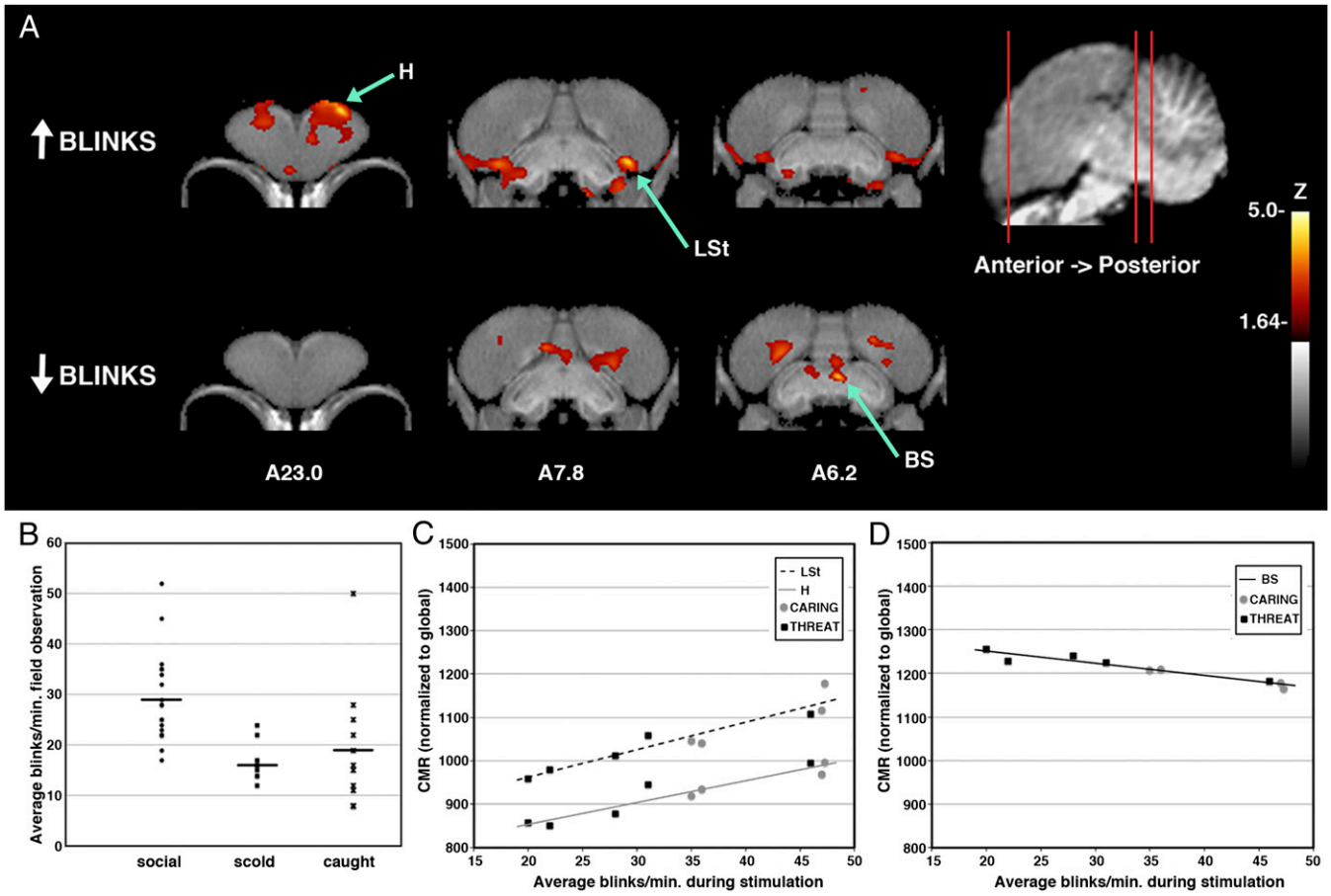

Fig. 5. Brain activation patterns associated with individual blinking behavior. (*A*) Voxel-wise linear regression with blink rates in crows shown a familiar face (threatening + neutral; $n = 9$; recorded during experiments) where the derived correlation coefficients were converted to *z*-score maps and superimposed to a structural MRI template of the crow brain for better anatomical localization. (*Upper*) Regions that were correlated positively with blink rates. (*Lower*) Regions where increased activation was associated with decreased blinking. Coronal slices (from anterior to posterior; coordinates refer to Japanese jungle crow atlas; ref. 31) illustrate peak activation in linear regression (voxels with $Z > 1.64$ are colored; those with $Z > 3.8$ are considered significant with associated structures as indicated). (*B*) Reduced blinking in nature by crows viewing threatening people (social, 19 crows averaged 29 blinks per min, SE = 2.0, while foraging with conspecifics; scold, 11 others averaged 16 blinks per min, SE = 1.1, as they scolded a threatening person; caught, 11 crows averaged 19 blinks per min, SE = 3.6, as we held them during capture; Kruskal–Wallis $H_{(2)} = 17.2$, $P < 0.001$). (*C*) Positive relationships between significant peak activation and blinking. Blinking rates were greatest for birds viewing the caring face (circles). All $r > 0.93$, $P < 0.0001$). (*D*) Negative relationship between significant peak activation and blinking. Blinking rates were least among crows viewing the threatening face (squares). All $r = -0.96$, $P < 0.00001$. H, hyperpallium; LSt, lateral striatum; BS, brainstem nuclei surrounding tractus occipitomesencephalicus, including locus coeruleus.

are completed, the animals can be returned to the wild. Understanding how wild animals integrate perception, memory, and emotion to behave adaptively may allow researchers to generalize important findings across species and sensory modalities, develop strategies to lower stress in captive animals, shape animal actions to reduce human–wildlife conflicts, and engage the public to appreciate the cognitive capacity of other species.

Methods

We captured crows lured from large roosting and foraging groups on three separate occasions using a netlauncher and selected only large (likely male), black-mouthed (adult) birds to bring into captivity (30). None of the birds had previously been captured, and there was no evidence (presence of previously banded birds) that any of the birds resided in study sites that we have previously used for research. Because multiple groups of crows were captured over the course of the study, we counterbalanced the masks used: The mask that was learned as threatening by some crows was learned as caring by others.

After capture, crows were housed for 4 wk in individual 1 × 2 × 2-m cages in accordance with Institutional Animal Care and Use Committee Protocol 3077-01, Washington Scientific Collection Permit 11-359, and US Scientific Collection Permit MB761139-1 (*SI Methods*). During this time, crows learned a new caring face, the mask worn at all times by the person feeding them and cleaning their cages. The threatening face was the mask used during the initial capture and when they were caught and moved to the PET laboratory. All masks were faces of actual people with neutral expressions; valence was conferred by our behavior, not by facial features.

The evening before imaging, the test subject was moved to a covered 0.5 × 0.5 × 1-m cage in the imaging facility to acclimate, undisturbed, overnight (Fig. 1 and *SI Methods*). In the morning, crows were blindfolded, removed from the covered cage, administered 1 mCi of FDG via i.p. injection, returned to the cage for a 2-min rest, and then shown the masked investigators in 1-min on/off blocks for 14 min. After the activation protocol, blindfolded crows were anesthetized with 3% isoflurane in oxygen with a flow rate of 300-800 mL/min and imaged. Activation and image acquisition timing were based on our data from dynamic imaging of an anesthetized crow (*SI Methods*), which indicated much faster brain FDG uptake and washout than is seen in mammals, including mice (31). Each crow was assigned a treatment at random and scanned only once, precluding within-subject analyses but eliminating possible confounding effects of prior experience with injection and anesthesia.

High-resolution FDG-PET images were acquired using a Siemens Inveon PET system for 10 min from 27- to 37-min post-FDG administration (Fig. S1) followed by an ∼13-min attenuation scan and then reconstructed by using 3D ordered subsets expectation maximization/maximum a posteriori to a spatial resolution of 2.5 mm.

We stereotaxically aligned images to a jungle crow (*Corvus macrorhynchos*) atlas (32), facilitated by structural MRI of one American crow. Nine affine parameters were estimated and applied to images, for consistent stereotactic transformation of scans from the same subject (33, 34). Alignment precision was estimated to be 2–3 mm. After normalizing to global values, significant regional differences in cerebral metabolic rate were determined by using automated voxel-wise subtraction and *Z*-statistic mapping (NEUROSTAT) (8). Correlation with blink rate was obtained by a voxel-wise linear regression across all subjects exposed to face stimulation (35). We considered *Z* values that were >3.8 statistically significant, controlling the type I error rate approximately at $P = 0.05$ for multiple comparisons in a modified Bonferroni correction commonly used in imaging research (36). Volumes-of-interest (VOIs) for structures with a *Z* score of >3.8 were applied to individual images, and values were compared across groups by using a *t* test.

We directly observed blinking at close range during experiments and with the aid of 10× binoculars in the field. In the laboratory, we counted each flash of the white nictitating membrane during each minute of stimulation and calculated the average of these as $n = 7$ counts per subject as the blink rate. We video-recorded laboratory trials, but resolution was insufficient to count blinking. From August 15 to 22, 2011, in the Seattle area or on nearby Vashon Island, we obtained up to five 1-min counts of individual wild crows blinking under three social settings: (*i*) as we held them during capture, (*ii*) eating food within a group of conspecifics and heterospecifics, and (*iii*) scolding a person who was close to the focal crow's offspring. As in the laboratory, we averaged all blink counts obtained on a single bird to determine the subject's blink rate. All blink rates were counted by J.M.M. to eliminate possible variation among observers.

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Northward range extension of an endemic soil decomposer with a distinct trophic position

Carol Melody and Olaf Schmidt

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Northward range extension of an endemic soil decomposer with a distinct trophic position

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Ecological niche theory asserts that invading species become established only if introduced propagules survive stochastic mortality and can exploit resources unconsumed by resident species. Because their transportation is not controlled by plant health or biosecurity regulations, soil macrofauna decomposers, including earthworms are probably introduced frequently into non-native soils. Yet even with climatic change, exotic earthworm species from southern Europe have not been reported to become established in previously glaciated areas of northern Europe that already have trophically differentiated earthworm communities of 'peregrine' species. We discovered established populations of the earthworm *Prosellodrilus amplisetosus* (Lumbricidae), a member of a genus endemic to southern France, in six habitats of an urban farm in Dublin, Ireland, about 1000 km north of the genus's endemic range. Not only was *P. amplisetosus* the dominant endogeic (geophagous) earthworm species in two habitats, it also occupied a significantly different trophic position from the resident species, as evinced by stable isotope ratio analysis. The suggested ability of this non-native species to feed on and assimilate isotopically more enriched soil carbon (C) and nitrogen fractions that are inaccessible to resident species portends potential implications of decomposer range expansions for soil functioning including C sequestration.

Keywords: decomposers; ecological niche; soil carbon; soil macrofauna; species introductions

1. INTRODUCTION

Scientists have long extolled the importance of beneficial soil macrofauna for soil ecosystem functioning. In particular, earthworms are a valued component of the soil fauna owing to their roles in decomposition, nutrient cycling and physical soil engineering [1]. However, in land areas not recolonized naturally by earthworms since the Late Quaternary glaciation, including some North American forests, recent invasions by non-native earthworms have had highly detrimental impacts on soils and ecosystems [2,3].

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In northern Europe, including Great Britain and Ireland, earthworms recolonized glaciated areas during the Holocene, probably through a combination of natural and anthropogenic mechanisms such as farming and trade [3–5]. Almost all earthworm species found (outdoors) in northern Europe belong to the 'peregrine' group of about 33 holarctic Lumbricidae species that are now dispersed widely in temperate regions [6]. By contrast, records in northern Europe of any of the numerous endemic, non-peregrine species from southern Europe are sporadic, and full establishment has not been documented [3–5].

Notwithstanding the small total species pool, northern European earthworm communities are ecologically diverse, with coexisting species representing all three ecological groups, i.e. the endogeics (mineral-soil dwellers), epigeic (surface-litter dweller) and anecic (deep-burrowers) [4]. It is unlikely that major food resources (e.g. litter, dung, high-quality soil organic matter) are left unconsumed by these earthworm communities [7]. According to the ecological niche theory advocating stochastic competitive community assembly [8], it is therefore improbable that invading species can become established from introduced propagules. Even if new species were to become established in this situation, then they would be unlikely to have negative ecosystem effects as severe as those observed in earthworm-free, North American habitats [2,9], unless their resource use would differ markedly from that of resident species.

Here, we report the discovery in Ireland of established populations of the endemic southern European earthworm *Prosellodrilus amplisetosus* (Lumbricidae, Annelida). We also test the hypothesis that the trophic niches (defined as the use of soil carbon (C) and nitrogen (N) resources) of *P. amplisetosus* and of native earthworm species differ, by comparing natural C and N stable isotope compositions.

2. MATERIAL AND METHODS

(a) *Earthworm survey*

We conducted an earthworm survey on Airfield farm, Dundrum, Co. Dublin (53°17′ N, 6°14′ W, 75 m elevation), a 13.5 ha educational, urban, mixed farm. In March 2011, we surveyed 11 habitats distributed across the farm: reseeded pasture, reseeded pasture margin, old pasture, old pasture margin, meadow, deciduous wood, coniferous wood, compost heap, manure heap, formal garden and vegetable garden. The approximate habitat locations on the farm are illustrated in the electronic supplementary material, figure S1.

We excavated six soil blocks (25 × 25 × 25 cm) in each of the 11 habitats and extracted earthworms by handsorting. Then, we used 2 l of a 0.2 per cent mustard oil (allyl isothiocyanate dispersed in isopropanol) solution to expel deep-burrowing earthworms from each pit. In addition, we sampled a transect from the margin into the reseeded pasture at 0.5, 1.5, 2.5, 5.0 and 10.0 m. Earthworms were weighed alive, fixed in 4 per cent formalin and adults identified to species level using [4], and [5] in the case of *P. amplisetosus*.

(b) *Isotope ratio analysis*

We revisited the reseeded pasture in July 2011 to sample earthworms at 2 m from the margin near the previous transect location. We sampled a single plot (50 × 50 cm) to avoid spatial variability. We focused on co-occurring endogeic species but also included *Aporrectodea longa* and *Lumbricus* spp., as primarily litter-feeding groups. Individuals were identified, allowed to evacuate their guts, weighed, freeze-dried and powdered. Five individuals of each species were used except *Ap. longa* for which only two specimens were available. Adults were used except for *Lumbricus* spp. (all juveniles) and *Aporrectodea caliginosa* (four juveniles, one adult). Worm tissues were analysed using an Elemental Analyser—Isotope Ratio Mass Spectrometer (Europa Scientific 20–20) at Iso-Analytical Ltd. (UK), and results are expressed in delta per mil (‰) notation. Analytical precision (s.d., $n = 5$) for powdered bovine liver reference material ($\delta^{13}C = -21.60‰$, $\delta^{15}N = 7.65‰$)

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Table 1. Number of earthworm species, mean total abundance and biomass (all earthworms, adults and juveniles) in each habitat ($n = 6$, s.d. in parentheses).

habitat	no. of species	individuals (m^{-2})	biomass ($g\ m^{-2}$)
reseeded pasture	12	635 (226)	195 (89)
reseeded pasture margin	10	555 (244)	140 (50)
old pasture	10	544 (105)	185 (61)
old pasture margin	7	184 (95)	91 (60)
meadow	9	504 (442)	107 (109)
deciduous wood	9	306 (136)	156 (102)
coniferous wood	4	115 (175)	72 (53)
compost heap	12	371 (514)	90 (125)
manure heap	6	138 (136)	38 (40)
formal garden	5	309 (84)	42 (20)
vegetable garden	9	171 (215)	36 (41)

was 0.02‰ and 0.06‰ for C and N. We used MANOVA to compare isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) of species, and MANCOVA of endogeic species data with individual worm biomass and C : N ratios as covariates (MINITAB v. 16) to rule out that the latter two factors caused artefactually distinct isotopic values [10].

3. RESULTS

We recorded high earthworm abundances (115–635 individuals m^{-2}) and species richness (4–12 species $habitat^{-1}$; table 1). On this one farm, we recorded 16 of the 27 lumbricid earthworm species found in Ireland (electronic supplementary material, table S1).

We recorded abundant *P. amplisetosus* populations in six different habitats, distributed across the western half of the farm (see the electronic supplementary material, figure S1), out of 11 habitats surveyed (figure 1*a*). In the case of the meadow and the reseeded pasture margin, *P. amplisetosus* was dominant among the endogeic species (figure 1*a*). The transect results (figure 1*b*) suggested a strong decline in *P. amplisetosus* numbers (absolute and relative) with distance from the hedgerow into the reseeded pasture field.

MANOVA revealed significant differences among the six cosampled species in $\delta^{13}C$ and $\delta^{15}N$ (Wilk's $F_{10,36} = 17.9$, $p < 0.001$). The C and N isotope biplot (figure 2*a*) showed that *P. amplisetosus* had a distinct trophic position compared with all other soil-feeding species and, when tested separately, was significantly more isotopically enriched than the nearest (and morphologically most similar) endogeic species, *Aporrectodea rosea* (Wilk's $F_{2,7} = 7.6$, $p < 0.05$). Litter-feeding *Lumbricus* spp., also showed an expected trophic difference (less enriched in ^{13}C and ^{15}N; figure 2*a*).

The distinct isotopic composition of *P. amplisetosus* among endogeics (figure 2*a*) was not simply an effect of body size (figure 2*b*), because juvenile *Ap. caliginosa* were smaller than *P. amplisetosus* but isotopically less enriched (by 0.71‰ and 2.29‰ in $\delta^{13}C$ and $\delta^{15}N$). The C : N ratio of *P. amplisetosus* was higher than that of the other endogeic species analysed (figure 2*b*), but neither body mass nor C : N ratio was a significant covariate in MANCOVA ($p > 0.10$).

4. DISCUSSION

All lumbricid species recorded in Great Britain and Ireland, including the few recent additions, are

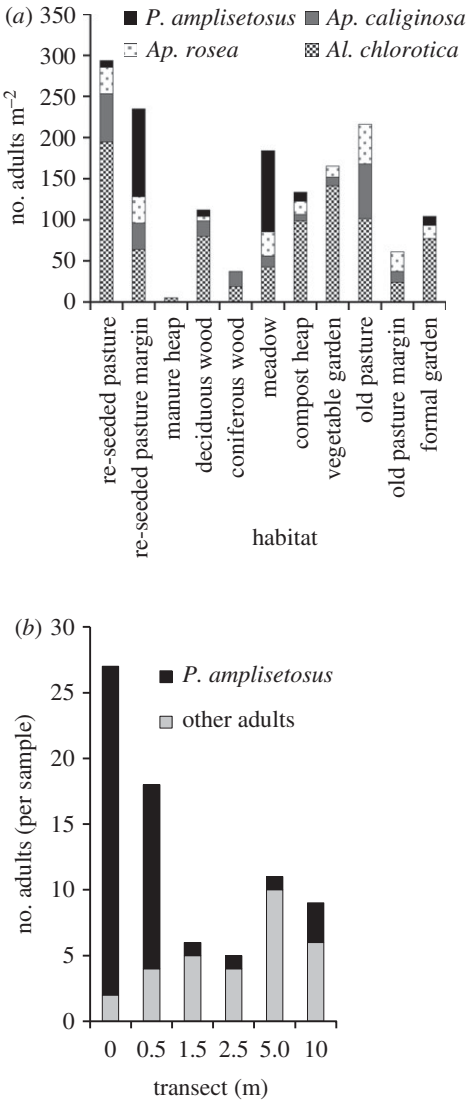

Figure 1. (*a*) Mean abundance (adults m^{-2}, $n = 6$) of the four endogeic earthworm species (*Ap. caliginosa*, *Allolobophora chlorotica*, *Ap. rosea* and *P. amplisetosus*) in 11 habitats. See table 1 for total abundances. (*b*) Number (adults) of *P. amplisetosus* and all other earthworm species (per 25 × 25 × 25 cm sample) from a transect in the reseeded pasture.

peregrine species or belong to peregrine genera [4,11]. By contrast, the genus *Prosellodrilus*, comprising about 25 species, has a small, endemic range in the Aquitaine region of southwestern France, with a few species occurring in northern Spain, Sardinia and northern Africa [5,12]. The established *P. amplisetosus* populations in Dublin represent a northward range extension for the genus of about 1000 km. A previous record of a single adult *P. amplisetosus* from a farm in County Louth, Ireland [13], together with sporadic records from single, agricultural locations in Galicia, Spain [14] and Burgundy, France [15], could suggest that this species' geographical range is expanding.

The extent and speed of northward range extensions, as well as the agents and climate factors, are well-documented for economically important taxa such as invasive terrestrial arthropods [16] and plant pests [17]. Soil decomposer species, by contrast, are not considered pests or a biosecurity risk, yet they are

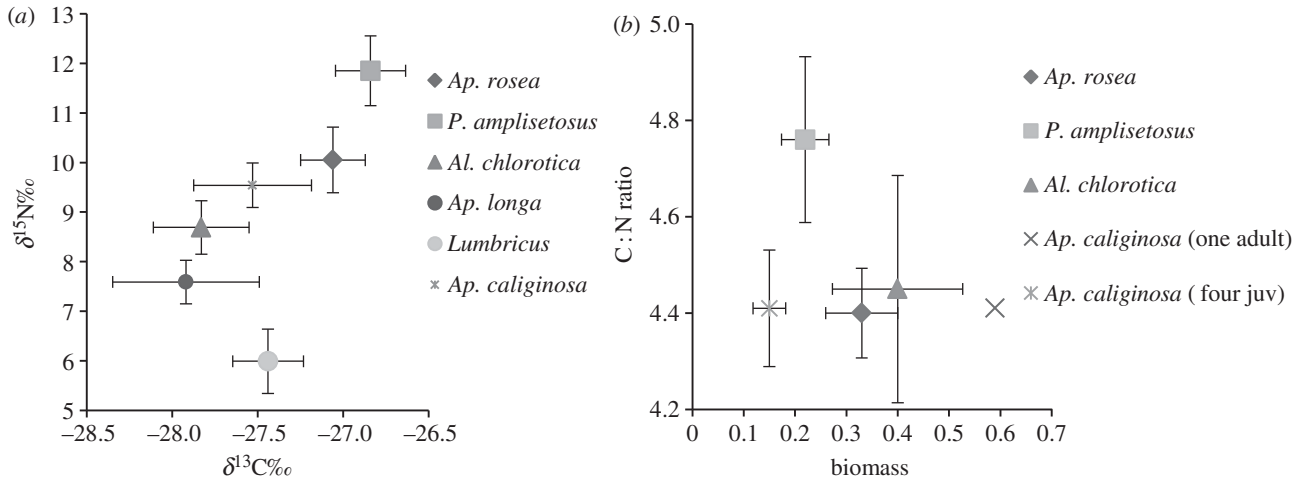

Figure 2. (*a*) Carbon (C) and nitrogen (N) stable isotope composition of five co-occurring earthworm species and juvenile *Lumbricus* spp. (means ± s.d., $n = 5$ except *Ap. longa* $n = 2$). (*b*) Mean biomasses and mean C : N ratios of the four endogeic species (mean ± s.d., $n = 5$ except *Ap. caliginosa* $n = 4$ juveniles, $n = 1$ adult).

likely to be transported frequently. The European earthworm distribution has not been mapped formally [11,12], and hence range shifts or expansions have not been documented systematically.

The high earthworm abundances and species richness observed at Airfield are comparable to those in similar habitats across Ireland [18]. The six habitats in which *P. amplisetosus* was recorded had between five and 12 native earthworm species, including three endogeic species (*Al. chlorotica*, *Ap. caliginosa* and *A. rosea*) that are among the most widespread and successful of all peregrine lumbricid species [5]. Stable isotope data suggest strongly that *P. amplisetosus* is the most extreme soil-feeder of all endogeic, geophagous species. Its isotopic elevation was not related to body size or biochemical composition; in fact the higher C : N ratio of *P. amplisetosus* tissues implied a higher lipid content and thus a more negative $\delta^{13}C$ value [10], and smaller body size in endogeic species was associated with lower $\delta^{15}N$ values in a previous study [10]. A possible mechanistic explanation for this distinct trophic position among endogeics is that *P. amplisetosus* assimilates C and N from other soil organic matter fractions. Perhaps it consumes soil organo-mineral particles containing soil organic matter fractions that are more microbially processed and hence more ^{13}C and ^{15}N enriched [19] than those consumed by resident endogeic species. A depth effect is unlikely in a reseeded (ploughed) pasture soil.

While the suggested exploitation of unused soil C and N resources by *P. amplisetosus* concurs with niche theory and explains this species' invasion success, it also has potential implications for soil functioning [9]. If soil decomposer taxa such as *Prosellodrilus* expand their ranges, facilitated by climatic change, they might access and mobilize soil organic C pools that are unavailable to resident species. Southern species could also stay active longer under drier conditions. This novel feeding activity could potentially counteract the beneficial effects of resident species [1] on C and N dynamics, soil C sequestration and also soil physical properties.

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RESEARCH ARTICLE

Survival and arm abscission are linked to regional heterothermy in an intertidal sea star

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SUMMARY

Body temperature is a more pertinent variable to physiological stress than ambient air temperature. Modeling and empirical studies on the impacts of climate change on ectotherms usually assume that body temperature within organisms is uniform. However, many ectotherms show significant within-body temperature heterogeneity. The relationship between regional heterothermy and the response of ectotherms to sublethal and lethal conditions remains underexplored. We quantified within-body thermal heterogeneity in an intertidal sea star (*Pisaster ochraceus*) during aerial exposure at low tide to examine the lethal and sublethal effects of temperatures of different body regions. In manipulative experiments, we measured the temperature of the arms and central disc, as well as survival and arm abscission under extreme aerial conditions. Survival was related strongly to central disc temperature. Arms were generally warmer than the central disc in individuals that survived aerial heating, but we found the reverse in those that died. When the central disc reached sublethal temperatures of 31–35°C, arms reached temperatures of 33–39°C, inducing arm abscission. The absolute temperature of individual arms was a poor predictor of arm abscission, but the arms lost were consistently the hottest at the within-individual scale. Therefore, the vital region of this sea star may remain below the lethal threshold under extreme conditions, possibly through water movement from the arms to the central disc and/or evaporative cooling, but at the cost of increased risk of arm abscission. Initiation of arm abscission seems to reflect a whole-organism response while death occurs as a result of stress acting directly on central disc tissues.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/216/12/2183/DC1

Key words: autotomy, body temperature, intertidal ecosystem, lethal temperature, *Pisaster ochraceus*, sublethal effect, thermal ecology, thermal heterogeneity.

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INTRODUCTION

The pervasive effects of an organism's body temperature have long attracted the attention of biologists. Temperature is among the most important abiotic factors driving the physiology and ecology of organisms (Hochachka and Somero, 2002), and a large amount of literature has accumulated on the ways that the physical environment interacts with an organism's physiology to drive its fundamental niche space (Kearney, 2006). The recognition of the ongoing and future impacts of climate change has motivated the application of concepts from thermal ecology and physiology to predicting the likelihood of changes in the distribution of organisms based on their thermal sensitivities and adaptations (Chown and Nicolson, 2004; Kearney and Porter, 2009; Buckley et al., 2010). Nevertheless, a conceptual mismatch has often occurred between thermal biologists, who typically recognize body temperature as the interface between organism physiology and environment, and most ecologists working on climate change issues, who generally use ambient air or sea surface temperature as an (often unrealistic) proxy of body temperature. The growing awareness of the importance of considering the actual body temperature experienced by organisms rather than simply relying on environmental correlates (Helmuth et al., 2010) has resulted in the development of heat budget models to predict body temperature variability over a wide range of spatial and temporal scales (Porter and Gates, 1969; Pincebourde et al., 2007; Kearney et al., 2009; Kearney et al., 2010). Biophysical studies have emphasized that body temperature varies not only as a function of microhabitat (Denny et al., 2011), but also with factors such as body size, which can affect an organism's thermal inertia (Turner, 1988; Helmuth, 1998; Pincebourde et al., 2009). These studies have also been used successfully to explore the impacts of environmental change in terms of growth and reproduction (Kearney et al., 2010; Schneider et al., 2010; Sarà et al., 2011), and rates of predation and thus interaction strengths between species (Sanford, 2002; Yamane and Gilman, 2009; Monaco and Helmuth, 2011).

However, while coupled heat budget and physiological models of large endotherms such as mammals have recognized the importance of within-body temperature heterogeneity (e.g. Porter and Gates, 1969), biophysical models of ectotherms often assume that temperature is uniform within the organism, or else that core temperature is the most important variable (e.g. Helmuth, 1998; Szathmary et al., 2009). However, for many organisms, body temperature can be heterogeneous at the within-organism scale, a

phenomenon called regional heterothermy. Temperature deviations between body regions have been observed in a wide variety of ectothermic organisms, including many insects (May, 1995; Coelho and Ross, 1996; Woods et al., 2005), some intertidal invertebrates (Fyhn et al., 1972) and reptiles (Garrick, 2008; Dubois et al., 2009). Within-body thermal heterogeneity has important physiological and ecological consequences. For example, some butterflies depend on regional heterothermy to 'pre-warm' flight muscles (Kingsolver, 1983), with significant implications for predator avoidance (Srygley and Chai, 1990). Some organisms have evolved sophisticated mechanisms to generate regional heterothermy. For example, in large ectotherms such as some reptiles, blood flow to appendages can be modified to control for warming or cooling rates (Dzialowski and O'Connor, 1999; Dzialowski and O'Connor, 2004). Flying insects such as bees or bumblebees keep their thorax hot and their abdomen relatively cool *via* continuous circulation of hemolymph between the two body regions through a counter-current heat exchanger, with heat being exchanged between outgoing and ingoing fluids (Heinrich, 1996). Liquid drop exudation to lower a body region temperature is another strategy used by mosquitoes (Lahondère and Lazzari, 2012) and some hymenopterans (Coelho and Ross, 1996; Heinrich, 1996). More generally, strategies involving heat exchangers (including exudation drops) to cope with sublethal temperatures might be widespread and are associated with within-body thermal heterogeneity.

Regional heterothermy can also be 'passive' in ectotherms, corresponding to the temperature difference between various regions of an organism's body due to variations in heating rates resulting from the specific mass, color or morphology of the body parts in interaction with the environment. The proximal causes for passive regional heterothermy are diverse. Differential patterns of wind flow over the surface of an organism can cause the appendages to remain cooler than the core (Tsuji et al., 1986). The orientation angle of different body regions to solar radiation can also generate a temperature gradient across the body (Kingsolver and Watt, 1983). Passive regional heterothermy has been studied notably in butterfly species during sun-basking behavior (Kingsolver and Moffat, 1982). Overall, however, regional heterothermy is likely to result from an association of both passive and active processes for most ectotherms. Recently, for example, the major claw of the fiddler crab was suggested to serve as a heat emitter, collecting the heat (active way) from the body exposed to solar radiation, and releasing it (passive way) to the environment *via* convection (Darnell and Munguia, 2011).

Within-body thermal heterogeneity raises important physiological and ecological questions that heretofore have received relatively little attention for most ectotherms. For example, how do we measure and model body temperature if temperature varies across the organism's body? Do upper lethal temperatures always correspond to the tolerance level of the most sensitive body region? What are the sublethal consequences of variation in body temperature? Can organisms regulate their within-body thermal heterogeneity to improve their resistance to high temperatures? Do mobile organisms decide to leave an unfavorable microhabitat based on the temperature of a specific body region? The rise of thermography technology, which is now accessible at moderate cost, offers a promising tool in this context. For example, infrared cameras can be used to measure directly, non-invasively and simultaneously the surface temperature of all body regions for a single or multiple individuals (e.g. Pincebourde et al., 2009; Lahondère and Lazzari, 2012), and biomimetic sensors (e.g. Lima and Wethey, 2009) could potentially be designed to record temperatures of different body regions over prolonged periods in the field, although this has yet to be attempted.

Here we investigate the within-body thermal heterogeneity in the sea star *Pisaster ochraceus* (Brandt 1835), a keystone predator known to exhibit behavioral and physiological responses to body temperature during aerial exposure at low tide (Pincebourde et al., 2008; Pincebourde et al., 2009; Pincebourde et al., 2012). Intertidal organisms are alternatively exposed to aerial and underwater conditions during tide cycles. At low tide, the body temperature of intertidal ectotherms can differ substantially from ambient air or surface temperature, and can fluctuate drastically over short time intervals (minutes), as it is driven by the interaction of multiple environmental factors (Helmuth, 1998; Wethey, 2002). Thus, intertidal organisms experience large thermal variations and their body temperatures can frequently reach sublethal levels (Somero, 2002). The predatory sea star *P. ochraceus* is quite sensitive to both submerged (Sanford, 2002) and aerial body temperature (Fly et al., 2012; Pincebourde et al., 2008; Pincebourde et al., 2009; Pincebourde et al., 2012). Transplant experiments in central Oregon reported high mortality when this species was experimentally kept high on the shore (Petes et al., 2008). Previous studies have shown an upper lethal body (central disc) temperature of 35°C and have demonstrated a significant negative influence of temperature on feeding rates at an aerial body (central disc) temperature of 23°C and above (Pincebourde et al., 2008). However, *P. ochraceus* can exert behavioral control over its body temperature in two ways. First, animals may avoid thermally stressful environments by remaining low in the intertidal zone, despite the presence of a significant food source (mussels) higher on the shore (Robles et al., 1995), or by exploiting shaded microhabitats (Fly et al., 2012). Studies along the west coast of North America showed that this species only rarely forages in environments that would lead to a low-tide body temperature above 29°C (Sanford, 2002; Pincebourde et al., 2008; Pincebourde et al., 2012; Broitman et al., 2009; Szathmary et al., 2009; Fly et al., 2012). This suggests that the exposure to sublethal body temperatures in the range of 29–35°C may incur high energetic costs or lead to significant physiological damage. Second, the sea star shows an interesting thermoregulatory feature in that it increases the internal fluid volume following exposure to aerial body temperatures of ~27°C. By increasing the thermal inertia of the body cavity, this behavior slows down heating rate during the next low tide exposure (Pincebourde et al., 2009). We investigated the hypothesis that exposure to sublethal body temperatures in the range of 29–35°C alters the sea star morphology through arm abscission, with the arms reaching higher temperatures than other body regions (central disc).

Previous studies of this sea star have focused on measuring (Pincebourde et al., 2008) and modeling (Szathmary et al., 2009) the temperature of the central disc, because it houses critical organs (e.g. nerve ring, stomach and core of water vascular system) and it is essential for regeneration of lost arms. Arm loss in *P. ochraceus* has been documented, although the causes remain unclear (Lawrence, 1992) (supplementary material Table S1). Asteroids (Phylum Echinodermata) have the ability to regenerate arms that are damaged by natural causes (e.g. predation) and lost *via* abscission (Lawrence, 1992). However, losing an arm represents a huge cost for the sea star in terms of energy and reproductive output (Lawrence and Larrain, 1994; Barrios et al., 2008), because arms contain both stored resources (pyloric caeca) and the gonads (Lawrence and Lane, 1982; Sanford and Menge, 2007). The relative importance of arm and central disc temperatures to lethality and arm abscission remains unclear, as does the possibility that trade-offs exist between prevention of arm loss and risk of mortality. We measured the within-body thermal heterogeneity of sea stars and compared the temperature of arms with that of the central disc under extreme aerial

conditions at low tide. We performed laboratory temperature assays to test whether sea star survival depends primarily on the temperature of the arms or the central disc, and specifically tested the hypothesis that arm abscission would be more closely associated with aerial arm temperatures than with central disc temperature. Then we focused on the survivors that lost one or several arms in these assays to determine whether trade-offs exist between short-term survival probability and arm abscission.

MATERIALS AND METHODS

Study system and organisms

The sea star *P. ochraceus* is common on rocky intertidal shores along the west coast of North America (Menge et al., 2004). All individuals were collected at low tide at a single study site near the Bodega Marine Reserve, California (38°19′N, 123°4′W). Wet body mass of individuals fell in the range 150–250 g (mean ± s.d.=193.1±30.9 g, *N*=70), which is the most common wet body mass range for this species in California (Menge et al., 2004). Animals were brought to the Bodega Marine Laboratory, where they were maintained submerged in a large tank with flow-through seawater. Animals were kept at a water temperature of ~13°C for 20 days before the experiment started. This acclimation period was set to homogenize the physiological state of all individuals.

Experimental design

The experiment was designed to measure the sublethal (arm loss) and lethal effects of aerial body temperature during low tide. Some of the data collected during this experiment (lethal central disc temperature) have been published elsewhere for a different purpose (Pincebourde et al., 2008). In this study, we tested whether arms can be damaged by elevated temperatures before the central disc (vital region) reaches the upper lethal temperature, and whether arm loss is most closely related to a whole-organism response or to the temperature of the arm itself. A single aquarium (75 l) was used to reproduce tidal conditions. Two heat lamps (150 W each) were positioned above the aquarium, and their height relative to the sea stars was adjusted to vary body temperatures during aerial exposure. Seawater was run continuously into the bottom of the tank beneath a flat, elevated platform that prevented sea stars from contacting the water, while keeping the relative humidity constant and high. Sea stars were placed in a flat position and were not overlapping with each other. They were exposed to aerial conditions for 6 h, corresponding to a typical aerial exposure during low tide at the study site (Pincebourde et al., 2008). The heat lamps were switched on after 1 h of exposure. The height of the lamps was then gradually decreased until experimental body temperature was reached after 3 h of aerial exposure. The experimental temperature (i.e. central disc temperature) was maintained during the last 3 h of the simulated low tide. Body region temperatures were checked every 15 min throughout the treatment using an infrared camera (ThermaCAM 695, FLIR Systems, Boston, MA, USA; thermal sensitivity <0.05°C, accuracy <1°C within the temperature range tested, spatial resolution 1.36 mrad). The infrared camera measures surface temperature of the aboral (dorsal) side, which is close (~1°C) to internal body temperature (~center of the disc) in the sea star *P. ochraceus* (Pincebourde et al., 2012), although gradients of several degrees between the aboral and oral surfaces have been observed (S.P., E.S. and B.H., personal observation). This setup generated significant thermal heterogeneity in different body regions, with the arms positioned close to the normal of the heat lamps reaching higher temperature than the other arms. We verified that this situation was representative of sea stars exposed to solar radiation by taking infrared photographs of sea stars under these conditions at low tide in the field. We found that mean arm temperature was 0.8 to 2.1°C higher than mean central disc temperature (*N*=10 individuals, in the central disc temperature range 14.0–23.7°C), which corresponds to the range of temperature deviations in our experiments. After 6 h of aerial exposure, a stand pipe was fixed in the aquaria to fill it completely with seawater, thereby simulating the high tide with water temperature ~13°C.

Experimental procedure

Ten groups of seven individuals each (*N*=70) were exposed to different experimental aerial body temperatures. Body temperatures varied slightly among individuals of the same group according to their position relative to the heat lamps. The heat lamp was set at a different height for each group, to ensure that sampling was evenly distributed across the thermal range, with a 2°C increment from 26 to 42°C. For each sea star, the central disc temperature was measured as the average of six spot measures taken with the infrared camera over the central disc during the last 3 h of aerial exposure. We also recorded the temperature of each arm by targeting the middle of the arms with the infrared camera. Arms were identified according to their position relative to the madreporite (the opening to the water vascular system on the aboral surface).

The water balance of individuals was also measured as it was expected to be influenced by variation in overall body temperature. An electronic scale was used to record wet body mass of sea stars (to the nearest 0.1 g), as an indirect measurement of variation in fluid volume (Pincebourde et al., 2009). Wet body mass was measured at both the start and the end of the experimental low tide (except for sea stars that were already dead at the end of treatment), to estimate the amount of water lost during the treatment. Subsequently, wet body mass was measured again after an 18 h period of submergence on sea stars that were alive at this time. This last measure was used to estimate the amount of body fluid recovered during the submergence period (Pincebourde et al., 2009).

Survival was assessed for all individuals at the end of the treatment and after a 24 h submersion period immediately following the experimental low tide. Each animal was placed on its dorsal (aboral) surface and a sea star was considered alive only when movement of the tube feet was observed 24 h post-treatment. Finally, the sea stars were put back into a holding tank for 3 weeks and the number and identity of arms lost were recorded. All individuals alive after 24 h were still alive after 3 weeks spent in the holding tank.

Data analysis

Six responses were measured for each individual: (1) survival, (2) temperature of the central disc, (3) temperature of each arm, (4) number and position of lost arms, (5) amount of water loss during the experimental low tide and (6) amount of water recovered at the end of the following high tide. The analysis followed three steps.

Firstly, we quantified the influence of body region temperatures on the overall water balance. Pearson correlations were performed to analyze the influence of central disc and arm temperatures on water loss and water recovery. Each variable of the water balance was expressed as a percentage of wet body mass. Three groups of individuals were distinguished: alive with no damage, alive with arm abscission, and individuals that were alive at the end of the experimental low tide (for water loss), or 18 h after this exposure (for water recovery), but which died later on.

Secondly, we analyzed survival rate as a function of arm and central disc temperatures and water balance to determine which

variable was a better predictor of survival. We also examined the relationship between arm and central disc temperature in animals that survived, animals that died and animals that survived with arm loss. Pearson correlations were performed to analyze the association between these variables. Then, logistic dose–response regressions were performed to quantify the effect of central disc temperature, arm temperature and water loss on survival.

Finally, we focused on the survivors (both with and without arm loss) to explore the sublethal effect of temperature. Dead individuals were therefore excluded from this analysis. We estimated the range of the sublethal zone in terms of mean arm temperature and mean central disc temperature by plotting the number of arms lost against these two temperature variables. A LOESS spline estimation analysis was performed using smoothed data to estimate the temperature threshold initiating damage to arms. Then, every arm of each individual was considered as a separate entity to perform a logistic dose–response regression in order to study the global relationship between the temperature of an arm and its probability of 'survival', i.e. whether the arm was lost. Next we considered the fate of arms at the intra-individual scale. For each individual, arms were ranked according to their temperature, from the coldest (rank=1) to the hottest (rank=5). We separated the individuals into three groups according to the number of arms they lost (one, two or three arms lost). A one-sample Kolmogorov–Smirnov test using uniform distribution was employed to determine whether the arms lost were consistently the hottest. All statistical tests were performed in SYSTAT 10 (Systat Software, Chicago, IL, USA) while regression analyses and LOESS procedures were performed using TableCurve 2D 5.01 (Systat Software).

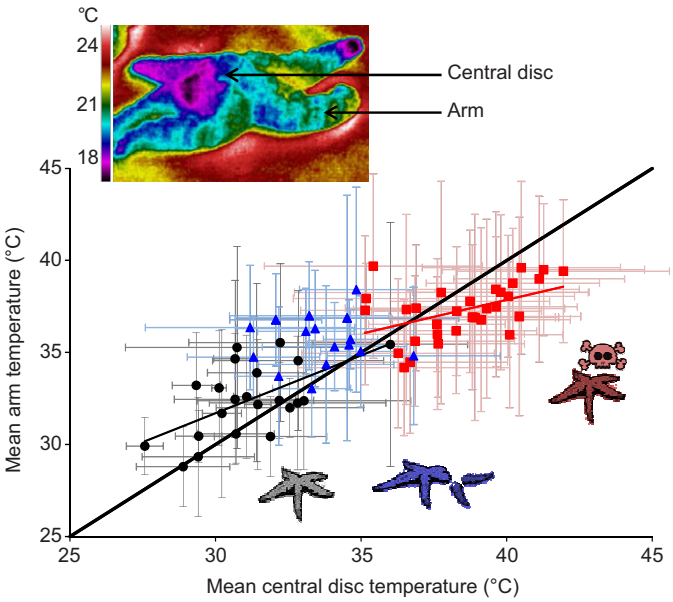

Fig. 1. Mean (±s.d.) arm temperature as a function of mean (±s.d.) central disc temperature for all *Pisaster ochraceus* individuals tested including the survivors with intact arms (black circles) and arm abscission (blue triangles), as well as the individuals that died (red squares). The linear regression lines are shown when correlations are significant. The black line indicates the equality of the two variables. The inset is an infrared image of a sea star during the exposure to experimental heating, illustrating the thermal heterogeneity of the sea star body surface (the color scale indicates temperature range).

RESULTS

Within-body thermal heterogeneity

The body temperatures of sea stars were heterogeneous with large deviations between body regions. The temperature deviation between the central disc and arms showed a distinct pattern depending on the temperature range (Fig. 1). For animals that survived with no arm loss, arms were on average warmer (29–35°C) than the central disc (27–33°C), but the temperature of the two body regions increased concomitantly (Pearson correlation: r=0.58, P=0.005). A similar correlation was also found in sea stars killed by the high temperature exposure (Pearson correlation: r=0.46, P=0.008), but in this case the arms were on average colder (34–40°C) than the central disc (36–43°C). However, the relationship did not hold for sea stars within the temperature range inducing arm abscission (Pearson correlation: P=0.83). In this category, mean arm temperature remained relatively constant (~35°C) over a range of central disc temperatures that were on average cooler (~31–35°C; Fig. 1). Overall, relatively large temperature deviations were found among arms and even within the central disc at the intra-individual scale (Fig. 1). This variability can be explained mostly by the various positions of the body relative to the heat source (lamps). Intra-individual variability increased slightly with both increasing central disc and arm temperatures throughout the temperature range tested (Pearson correlation on coefficients of variation; on arms: r=0.27, P=0.02; on central discs: r=0.41, P=0.001).

The effect of temperature on water balance

As expected, water loss during the experimental low tide was clearly influenced by temperature. Individuals with the warmest body lost up to 30% of their wet body mass during the treatment (Fig. 2A,B) and rate of water loss was significantly correlated with both central disc and arm temperature (Pearson correlation: r=0.80 and 0.64, respectively, P<0.001 for both). However, central disc temperature was a better predictor of overall water loss when comparing the residuals from a linear regression (mean-square of residuals from linear regression: 14.84 and 24.04 for central disc and arms, respectively). Similarly, the amount of water recovered during the following high tide was inversely related to central disc temperature (Pearson correlation: r=–0.82, P<0.0001) and arm temperature (Pearson correlation: r=–0.73, P<0.0001) (Fig. 2C,D). Central disc temperature was only a slightly better predictor of water recovery than arm temperature (mean-square of residuals from linear regression: 9.04 and 13.21 for central disc and arms, respectively).

Survival and arm loss rate were related to rates of water recovery. Most of the individuals that survived with no arm abscission increased their wet body mass by 2–6% following resubmersion, which is in agreement with the values given in Pincebourde et al. (Pincebourde et al., 2009). By contrast, individuals that lost one or several arms were not all able to recover initial wet body mass during the initial 18 h before they lost arm(s), with a change down to −7% (Fig. 2C,D). Finally, the individuals that subsequently died (between 18 and 24 h after treatment) showed the most dramatic decrease in wet body mass after the high tide, between −6 and −11% of initial wet body mass. The effect of water loss during aerial exposure was not as straightforward. Animals that lost the least amount of water (5–15%) were most likely to survive (Fig. 2A,B). However, there was no difference observed in the amount of water lost (10–30%) between animals that lost arms and animals that died, although both lost more water than survivors without arm loss.

The lethal effect of temperature

The logistic dose–response model using central disc temperature as the explanatory variable provided an estimate of the lethal temperature at 35.3°C (R^2=0.83, $F_{2,66}$=172.5, P<0.001), as has been

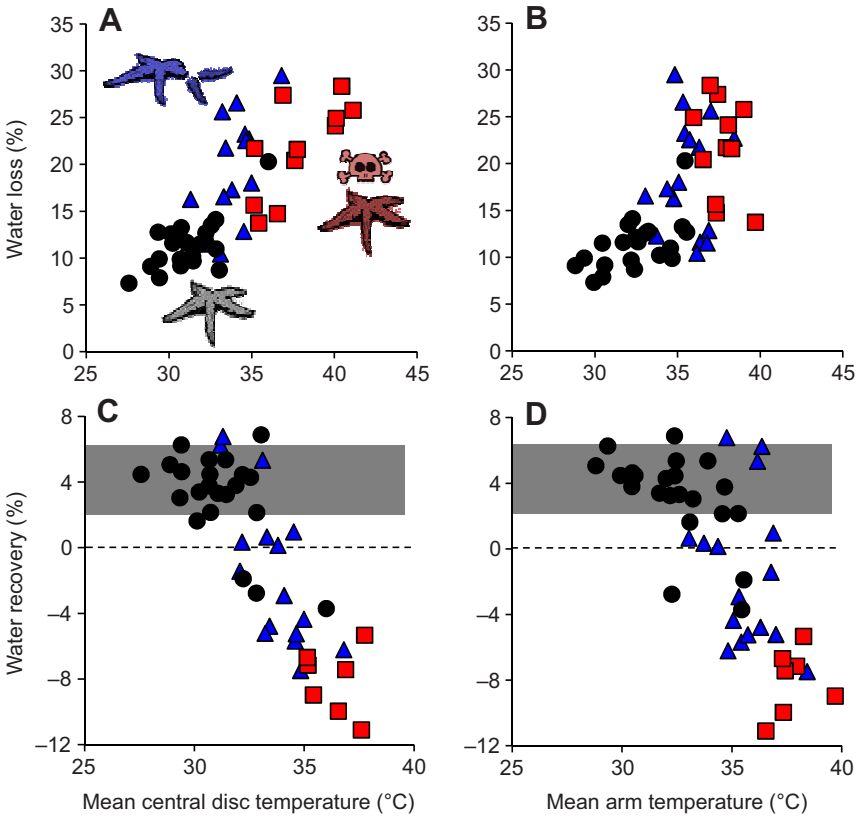

Fig. 2. Temperature influence on water balance in *Pisaster ochraceus*. (A,B) Water loss (% of initial wet body mass) during low tide as function of mean central disc temperature (A) and mean arm temperature (B). (C,D) Water recovery (% of initial wet body mass) during following high tide as function of mean central disc temperature (C) and mean arm temperature (D). The relationship is shown for survivors with no arm damage (black circles), survivors with arm abscission (blue triangles) and the individuals alive at the time of measurement that died subsequently (red squares). The gray bars indicate the expected water recovery portion (4.5±2.3% of initial wet body mass; mean ± s.d.) according to Pincebourde et al. (Pincebourde et al., 2009).

previously reported in Pincebourde et al. (Pincebourde et al., 2008). This thermal limit was very clearly identified, as there was little overlap between the temperatures at which animals survived and those at which they died (temperature range of the overlap: 1.6°C; Fig. 3A). This overlap was much greater, however, when the model used mean arm temperature (R^2=0.41, $F_{2,66}$=25.5, P<0.001; temperature range of the overlap: 4.5°C; Fig. 3B). The mean arm temperature regression model estimated the lethal temperature limit at 36°C. Overall, the logistic dose–response regression analysis showed that central disc temperature was a better predictor of survival probability than mean arm temperature (as the regression model using central disc temperature and mean arm temperature explained 83% *versus* 41% of the variability, respectively; Fig. 3A,B). Mortality was clearly caused by a direct effect of temperature rather than by a direct effect of water loss. Indeed, survival was not related to water loss during the experimental low tide ($F_{2,45}$=1.38, P=0.26; Fig. 3C), which is in accordance with a previous study that found that *P. ochraceus* is quite tolerant of desiccation (Landenberger, 1969).

The sublethal effect of temperature

The pool of survivors (N=38) was used to analyze the effects of exposure to sublethal temperatures on arm damage. Among these 38 survivors, five, six and five individuals lost one, two and three arms, respectively. Arm abscission was initiated at a mean arm temperature of ~32.5°C (LOESS spline estimation from smoothed data: tension 0.40, R^2=0.60, s.e.m.=0.76; Fig. 4A), corresponding to a mean central body temperature of ~30.5°C (LOESS spline estimation from smoothed data: tension 0.40, R^2=0.67, s.e.m.=0.67; Fig. 4B). The logistic dose–response model with arm temperature as the explanatory variable described poorly the loss of an arm when considering each arm as an independent entity (R^2=0.29, $F_{2,187}$=40.8, P<0.001, temperature range of the overlap: 13.1°C; Fig. 5). This model estimated a 'lethal' temperature for arms at 38.8°C. Although the specific temperature experienced by individual arms was a poor predictor of arm loss, the arms that were lost by an individual sea star were consistently among its hottest arms, whatever the total number of arms lost (one-sample Kolmogorov–Smirnov test using uniform distribution: P=0.03 for one and two arms lost, and P=0.001 for three arms; Fig. 6).

DISCUSSION

Exposure to sublethal temperatures can induce morphological responses, mostly temporary and reversible changes in morphology (e.g. posture or shape), to decrease body temperature in slow-moving organisms that cannot readily relocate to a new microhabitat (Garrity, 1984; Williams et al., 2005). Here we show that exposure to sublethal temperatures can lead to dramatic and long-lasting effects on sea star morphology by inducing arm abscission. Our results provide experimental evidence, for the first time in an echinoderm, of a direct relationship between temperature of body regions and arm abscission. All arm abscission events were observed with a separation plane located at the ~10th pair of ambulacral ossicles. Abscission always involved basal detachment of arms and we never observed distal damage on arms due to the heating treatment. This observation supports the hypothesis that arm abscission caused by high temperature exposure corresponds to a mechanism of autotomy in *P. ochraceus* initiated as a whole-organism response as opposed to local damage (but see below). However, arm abscission was delayed following exposure to thermal stress and was usually observed >2 days after the treatment. In general, in echinoderms (including asteroids), autotomy involves rapid (within at most a few minutes) abscission of a body part mediated by the nervous system, followed by a long regeneration

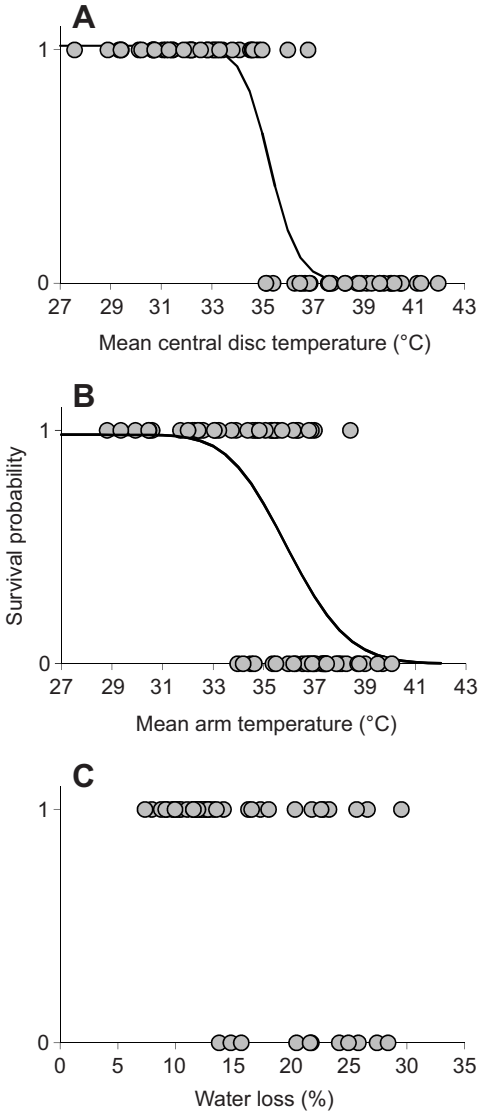

Fig. 3. Lethal effects of temperature on *Pisaster ochraceus*. Survival probability (1: survived, 0: died) of individuals according to mean central disc temperature (A) (from Pincebourde et al., 2008), mean arm temperature (B) and water loss (C). Curves indicate the logistic dose–response regression model.

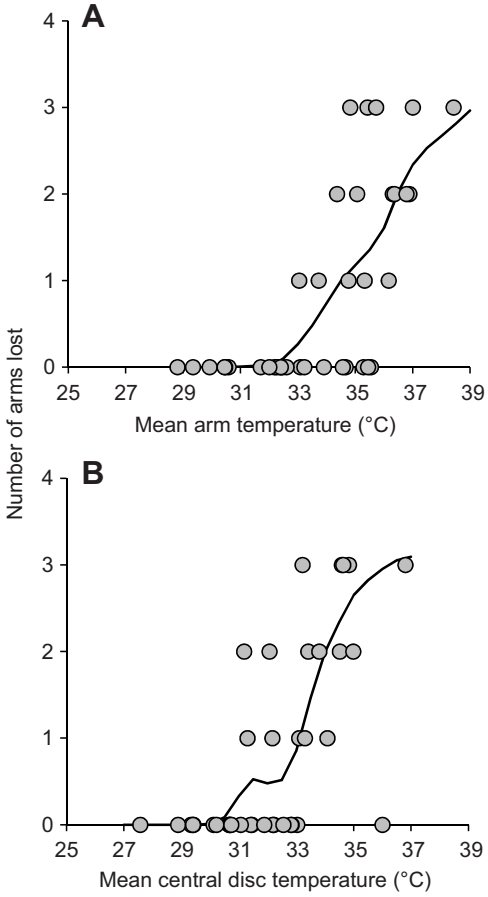

Fig. 4. Sublethal effects of temperature on *Pisaster ochraceus*. Number of arms lost in survivors as a function of their mean arm temperature (A) and mean central disc temperature (B). Curves illustrate the LOESS spline estimation from smoothed data with a tension of 0.40.

process (Mladenov et al., 1989; Marrs et al., 2000; Wilkie, 2001). Indeed, the incidence of arm loss in nature is rather infrequent in *P. ochraceus* (Lawrence, 1992), although regenerating individuals can be found in every population. Field surveys of *P. ochraceus* populations at five sites on the central Oregon coast revealed that 0.44 to 5.2% of the individuals at these sites were regenerating arms (supplementary material Table S1). Although the majority of *P. ochraceus* that were regenerating arms at these sites were juveniles (mean ± s.e.m. wet mass=65.6±9.66 g), 33% weighed >90 g and thus were likely sexually mature (Menge, 1974). In general, arm abscission by autotomy is thought to have evolved as a mechanism to escape from predation or as a means of renewing damaged or infected body tissues (Lawrence, 1992). To the best of our knowledge, the role of temperature as an elicitor or proximal cue of arm abscission and/or autotomy has not been tested experimentally before.

The distribution of heat throughout the sea star body shows that arms are generally warmer than the central disc within the temperature range that *P. ochraceus* can tolerate. From a biophysical standpoint, one might expect arms to heat up and cool down more rapidly than the central disc due to a higher surface area to volume ratio and a lower thermal inertia. Asteroids have a relatively large body cavity (coelom) that can be filled with fluid, thereby altering the heating rate of the central disc especially when the seawater is colder than the body temperature of the sea star in air, which is typically the case along much of the geographic range of this species. Indeed, the water balance of *P. ochraceus* is primarily altered in response to the central disc temperature, likely because the volume of fluids within the coelomic cavity is playing an important role in adjusting the thermal inertia of the sea star (Pincebourde et al., 2009). The central disc is the vital region of the sea star, and it is expected that survival is primarily related to temperature of this body region. Furthermore, appendages or peripheral body regions are usually cooler than the core in animals because of higher convective loss, although the reverse can be true when appendages act as solar collectors, as in butterflies. In *P. ochraceus*, arms are therefore expected to be cooler than the central disc at thermal equilibrium when assuming that heat is exchanged only passively between body regions and the environment. But our observations contradict this expectation.

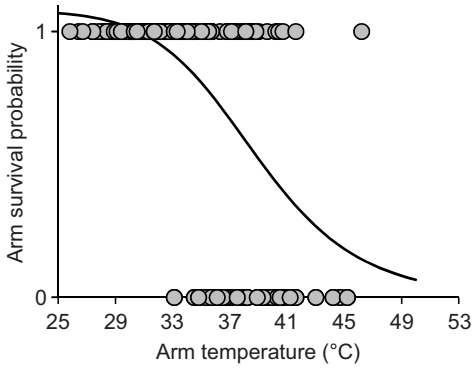

Fig. 5. Sublethal effects of temperature on *Pisaster ochraceus*. Survival probability of an arm when pooling all arms from survivors (1: intact arm, 0: arm loss) as a function of their temperature. The curve indicates the logistic dose–response regression model.

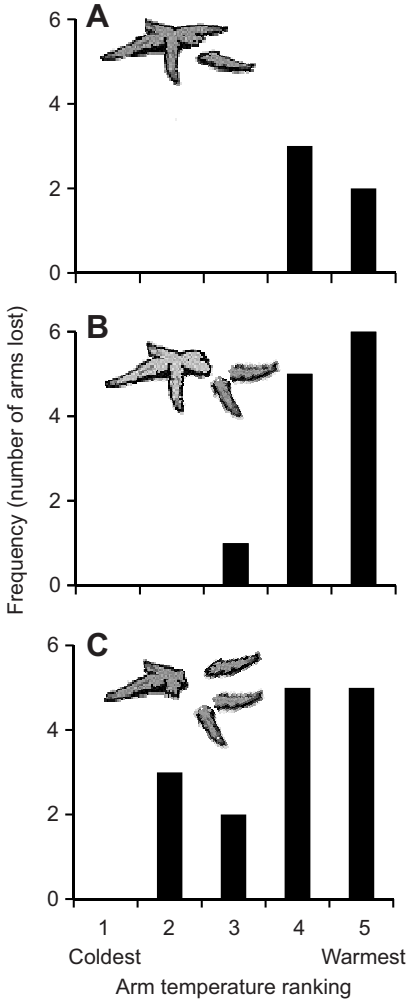

Fig. 6. Sublethal effects of temperature on *Pisaster ochraceus*. Temperature ranking of arm lost at the intra-individual scale (rank 1, coldest of the five arms, to rank 5, the hottest arm) for the groups that lost one (A), two (B) or three arms (C) after exposure to the heating treatment (*N*=5, 6 and 5 individuals in each group, respectively).

An intriguing hypothesis to explain the temperature deviation between body regions is that the arms might function as heat sinks, as proposed for the major claw in fiddler crabs (Darnell and Munguia, 2011). Such a mechanism implies a capacity to transfer heat between the central disc and the arms, where the heat can be exchanged more easily with the environment due to the comparatively higher surface area to volume ratio. Under this scenario, directional movement of fluid within the body might facilitate the transfer of heat from right below the dorsal integument, where solar energy is collected, to the arms where heat can be released to the environment *via* convective heat transfer. Directional flow can occur in the water vascular system (Prusch, 1977), but it is not known whether directed flows occur within the coelomic body cavity, although it is lined by ciliated epithelium. Another possible mechanism, not mutually exclusive from the hypothesis of arms acting as heat sinks, involves the potential for evaporative cooling. Evapotranspiration can occur across the dermal papulae, which are thin surfaces in the integument that are extensions of the fluid-filled coelomic body cavity. These structures explain why the integument of *P. ochraceus* is often wet at low tide. If fluid loss occurs disproportionately across papulae located on the central disc, then evaporative cooling might be greater in this vital region. The massive water loss we observed in individuals under high thermal stress (Fig. 2B) supports such a link between heterothermy and water balance, and can be interpreted as an emergency measure to cool down the body as its temperature approaches the tolerance threshold. Overall, whatever the mechanism, the observation that arms were cooler than the central disc in animals that died, and conversely that arms were warmer than the central disc in animals that survived without damage, supports the hypothesis of an active mechanism underlying the heterogeneity of body temperature. Nevertheless, little is known regarding such potential mechanisms in sea stars, and more detailed studies are needed to confirm the physiological and/or behavioral mechanisms that generate within-body thermal heterogeneity.

These hypothetical strategies, however, come with the risk of arm abscission, which incurs extremely high costs in terms of both energy and reproductive output (Lawrence and Larrain, 1994; Barrios et al., 2008), because the pyloric caeca and gonads are held within each of the arms. Although no wounds were observed externally on abscised arms, it is possible that arm loss was influenced by internal and irreversible damage caused by temperature, directly or indirectly. Indeed, abscised arms were more likely damaged by local desiccation rather than by a direct effect of temperature as we did not find a strong relationship between abscission and arm temperature (Fig. 5). Abscission might be favored by the advantages of retrieving fluids from a given arm (i.e. to be used for water balance management in the central disc), rather than triggered by a stress signal directly linked to temperature. This notion of sacrifice remains to be explored.

In general, beyond butterflies and some hymenopterans, little is known regarding how most ectothermic organisms regulate the thermal heterogeneity of their body regions during sublethal temperature exposure. We found large temperature deviations (5–10°C) between the central disc and the five arms in the sea star *P. ochraceus*. Our setup simulated the various angles of solar exposure to different body regions that can be found in the field. Thus, the thermal heterogeneity we describe can be initiated just by the presence of a heat source (solar radiation). Indeed, our infrared photographs confirm that within-body thermal heterogeneity occurs in *P. ochraceus* in the field at low tide. The within-body thermal heterogeneity of *P. ochraceus* is comparable to, or greater than, that of lizards [~4°C (Garrick, 2008)], bees [~10°C (Roberts and

Harrison, 1999)], mosquitoes [~4°C (Lahondère and Lazzari, 2012)] and triatomine bugs [~8°C (C. Lahondère, personal communication)] when feeding on hot blood, and even plant leaf surfaces [~4°C (Jones, 1999)]. Taken globally, these comparisons suggest that body surface thermal heterogeneity occurs over a wide range of body sizes and morphologies. The mechanistic link between this thermal heterogeneity and the physiological or behavioral response of many ectotherms to environmental stress is largely unexplored, but potentially important. The heat sink function of appendages is relatively frequent in vertebrates such as the enlarged ears of jackrabbits (Hill et al., 1980) and elephants (Weissenböck et al., 2010), or the bill of some birds (Tattersall et al., 2009), but this phenomenon remains understudied for most ectotherms (e.g. Darnell and Munguia, 2011).

Ecologists are now beginning to integrate body temperature into ecological niche models (Kearney and Porter, 2009; Kearney et al., 2010; Monaco and Helmuth, 2011). We suggest that understanding and characterizing within-body temperature heterogeneity is also quite important for at least three reasons. First, the effect of thermal exposure can potentially be a body-region-specific response to temperature or a whole-body response. Temperature can act directly on specific organs, cells, proteins or membranes, which are then identified as 'weak links' (Somero, 2002), or it can induce a global (physiologically adaptive) response. In *P. ochraceus*, the lethal effect appears to be a central-disc-specific response of temperature acting directly on this body region. By contrast, the sublethal effect (arm abscission) is a whole-organism response to temperature because there is not a specific temperature threshold for arm abscission induction and also because the identity of the arm that will be lost depends on its temperature relative to the others. Thus, the sublethal effect cannot be understood without knowledge of the within-body thermal heterogeneity. The way organisms integrate temperature changes in their environment, relative to their vital body regions, cannot be elucidated without this knowledge. Second, physiological rates measured in the laboratory might be only weak estimates of actual rates in the field unless a realistic heat source is used (Marshall et al., 2010). The presence and the quality of the heat source are crucial to mimicking the within-body thermal heterogeneity observed in the field. The metabolic rate of ectotherms, at the cellular or tissue scales, depends primarily on temperature. Therefore, temperature deviations between body regions could potentially translate into differences in their specific metabolism (e.g. assimilation and respiration). Different physiological functions can be optimized at different body temperatures; this is well known in reptiles (Huey and Kingsolver, 1989). Nonetheless, it is not clear whether ectotherms can adjust their within-body temperature heterogeneity to maintain several physiological functions near their optimal temperature in different body compartments. Third, ecologists have developed physical models that are useful in mapping body temperatures across space and time (Bakken and Gates, 1975; Fitzhenry et al., 2004; Dzialowski, 2005; Angilletta, 2009). These biomimetic loggers give estimates of body temperature, i.e. an average temperature over the entire body. Such a logger has been developed for the sea star *P. ochraceus* and has been shown to estimate the average temperature of the central disc (Pincebourde et al., 2008; Szathmary et al., 2009). Nevertheless, these loggers are unlikely to capture the thermal properties of peripheral body parts such as arms, legs or other appendages (e.g. crab claws), unless they are specifically designed with this question in mind. We recommend careful analysis of within-body thermal heterogeneity when building and testing such physical models. Overall, we suggest that integrating within-body thermal heterogeneity into biophysical models of ectotherm energy budgets will likely provide new insights into the responses of organisms to environmental stress and climate change.

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AUTHOR CONTRIBUTIONS

All three authors contributed significantly to conception, design and execution of the study, interpretation of the findings, as well as drafting and revising the article.

COMPETING INTERESTS

No competing interests declared.

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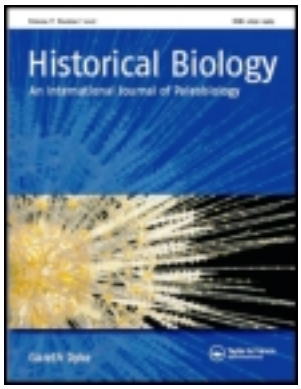

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Predatory behaviour of the social orb-weaver spider, Geratonephila burmanica n. gen., n. sp. (Araneae: Nephilidae) with its wasp prey, Cascoscelio incassus n. gen., n. sp. (Hymenoptera: Platygastridae) in Early Cretaceous Burmese amber

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Predatory behaviour of the social orb-weaver spider, *Geratonephila burmanica* n. gen., n. sp. (Araneae: Nephilidae) with its wasp prey, *Cascoscelio incassus* n. gen., n. sp. (Hymenoptera: Platygastridae) in Early Cretaceous Burmese amber

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The present work shows predatory behaviour of the social orb-weaver spider, *Geratonephila burmanica* n. gen., n. sp. (Araneae: Nephilidae) against a parasitic wasp, *Cascoscelio incassus* n. gen., n. sp. (Hymenoptera: Platygastridae) in Early Cretaceous Burmese amber. An adult male and juvenile of *G. burmanica* in the same web provide the first fossil evidence of sociality in spiders. The spider is characterised by a pedipalp with a hemispherical tegulum, a subtegulum curved at 180°and an apical spiralled embolas-conductor bent approximately 45°at midpoint. The male wasp is characterised by an ocellar tubercle, 12-segmented antennae with a feeble five-segmented clava, thick sensilla trichodea curvata with rounded ends on the claval antennomeres, a short uncus, a short post-marginal vein and a nebulose radial sector (Rs) vein extending from the uncus to the costal margin of the forewing. This is the first fossil evidence of spider sociality and a fossil spider attacking prey trapped in its web.

Keywords: Burmese amber; nephilid spider; platygastrid wasp; Cretaceous; *Geratonephila burmanica* n. gen., n. sp; *Cascoscelio incassus* n. gen., n. sp

Introduction

Fossil evidence of associations between predators and their prey is extremely rare (Boucot 1990; Boucot and Poinar 2010). Although amber contains many examples of insects captured in spider webs (Wunderlich 2004), there is no previous fossil record of a spider attacking its ensnared prey.

A piece of Early Cretaceous Burmese amber containing a spider web with a male and a juvenile spider as well as two captured insects (a neuropteran and a parasitic wasp) provides new insights into fossil predator–prey associations and sociality in spiders. The interacting biota offers an example of frozen behaviour by portraying an event from 100 million years ago (mya). The present study describes the spider and entrapped parasitic wasp, characterises the spider web and discusses sociality in spiders.

Materials and methods

The amber piece (Figure 1) is roughly rectangular in shape with a greatest length of 12.0 mm, greatest width of 5.4 mm and greatest depth of 3.0 mm. The amber was obtained from a mine first excavated in 2001, in the Hukawng Valley, south-west of Maingkhwan in Kachin State (26°20′N, 96°36′E) in Burma (Myanmar). This Noije Bum 2001 Summit site was assigned to the Early Cretaceous, Upper Albian, on the basis of paleontological evidence (Cruickshank and Ko 2003), placing the age at 97–110 mya. Nuclear magnetic resonance spectra and the presence of araucaroid wood fibres in amber samples from the Noije Bum 2001 Summit site indicate an araucarian (possibly *Agathis*) tree source for the amber (Poinar et al. 2007). Observations, drawings and photographs were made with a Nikon SMZ-10 R stereoscopic microscope and Nikon Optiphot compound microscope with magnifications up to 600×. Spider terminology follows that of Levi (2005) and Coddington (1990). Wasp terminology follows that of Masner (1976, 1980) and Masner et al. (2007). Higher wasp classification follows that of Sharkey (2007).

Descriptions

Spider

Family Nephilidae Simon, 1894

Geratonephila Poinar, new genus

Diagnosis: ovoid opistosoma; body and legs densely setose; male pedipalp with hemispherical tegulum; subtegulum curved 180°; apical spiralled embolas wrapped by conductor long, curved approximately 45°at midpoint.

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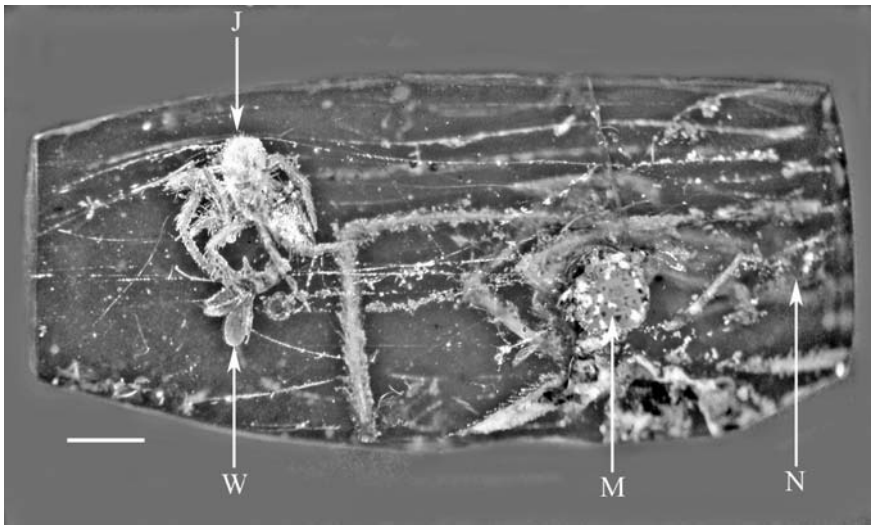

Figure 1. Entire piece with juvenile (J) and adult male (M) *G. burmanica* spiders together with neuropteran (N) and *C. incassus* wasp (W) prey in Early Cretaceous Burmese amber. Bar = 1.05 mm.

Type species: *Geratonephila burmanica* Poinar

G. burmanica Poinar, n. sp. (Figures 1–3)

Material examined: Holotype male and juvenile.

Description: Adult male. Length = 3.12 mm; width = 1.24 (ratio, 2.52); legs long, especially femora, tibiae and metatarsi of leg 1; walking leg formula, 1243; legs bearing both macrosetae and short setae; leg hair tufts absent; profemur, 3.30 mm; protibia, 3.24 mm; prometatarsus, 2.35 mm; rows of spines on legs; male pedipalp with hemispherical tegulum; subtegulum curved 180°; apical spiralled embolas wrapped by conductor, curved at midpoint approximately 45°; length bulb, 323 μm, width bulb, 409 μm, length conductor, 540 μm; opistosoma ovoid, longer than wide, length, 1.76 mm, width, 1.24 mm.

Juvenile: Length, 1.4 mm; carapace wider than long; length carapace, 360 μm, width carapace, 410 μm; opistosoma ovoid; length opistosoma, 820 μm, width opistosoma, 620 μm; legs and body with dense covering of thick, setae.

Type: Holotype deposited in the Buckley amber collection (accession No. E-2).

Type locality: Amber mine at the Noije Bum 2001 Summit site in the Hukawng Valley, south-west of Maingkhwan in Kachin State (26°20′N, 96°36′E) in Burma (Myanmar).

Figure 2. Juvenile *G. burmanica* in contact with its wasp prey, *C. incassus*, in Early Cretaceous Burmese amber. Bar = 440 μm.

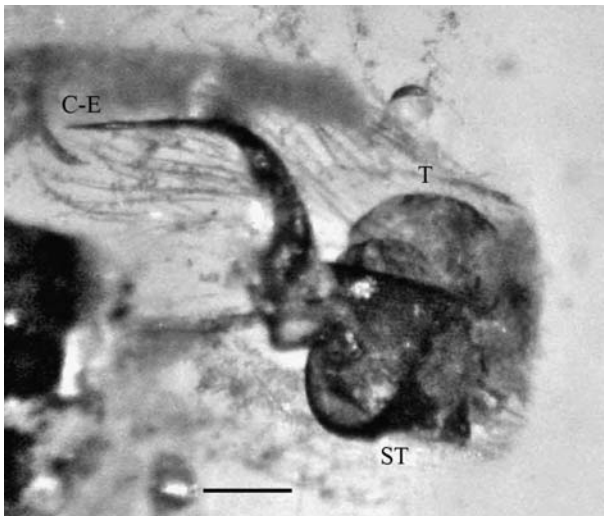

Figure 3. Pedipalp of male *G. burmanica* in Early Cretaceous Burmese amber. C–E represents conductor with embolus; ST represents subtegulum; T represents tegulum. Bar = 140 μm.

Etymology: The generic name is composed of the Greek 'geratos' for old, in reference to the age of the fossil and the type genus of the family, *Nephila*.

Comments: The Nephilidae is currently composed of two subfamilies (Nephilinae and Clitaetrinae) with 58 species in four genera (Platnick 2011). Since many characters on the fossil spiders are obscured, it is not possible to assign them to an extant or extinct genus. This is the first member of the Nephilidae described from Burmese amber; however, the family is fairly ancient with fossils dating back to the Jurassic (Selden et al. 2011).

Wasp

The wasp is complete and perfectly preserved in spite of its capture in the spider web.

Family Platygastridae

Cascoscelio Poinar, new genus

Diagnosis. Ocelli positioned on tubercle; weakly differentiated clava comprising terminal five antennomeres; thick sensilla trichodea curvata (STC) with rounded ends on claval antennomeres; forewing with long, complete submarginal vein reaching wing margin; short postmarginal vein subequal to marginal vein; stigma vein subequal to marginal and postmarginal veins; short uncus directed towards apical wing margin; nebulose Rs vein extending from uncus to costal margin of forewing; nebulose anal, cubitus and medius veins, of these only cubitus vein meets wing margin; marginal cilia fairly long; hind wing with submarginal vein complete, reaching costal margin beyond two frenal hooks; with long marginal cilia.

Type species *Cascoscelio incassus* Poinar

Etymology: Casco is from the Latin 'cascus' for old.

Cascoscelio incassus Poinar, new species (Figures 1, 2, 4–8)

With characters listed in the generic diagnosis.

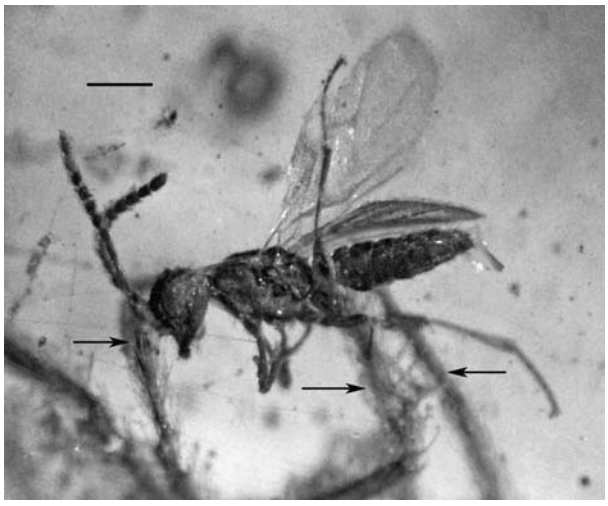

Figure 4. Habitus of *C. incassus* in Early Cretaceous Burmese amber. Arrows show spider legs in contact with the captured wasp. Bar = 240 μm.

Material examined: Holotype male

Description: Length, 1.2 mm; body and antennae brown; body shiny, with scattered, long setae (Figure 4).

Head: Length, 336 μm; frons depressed; compound eyes large, bare, oval, greatest diameter 160 μm; ocelli positioned on tubercle; lateral ocelli positioned closer to compound eyes

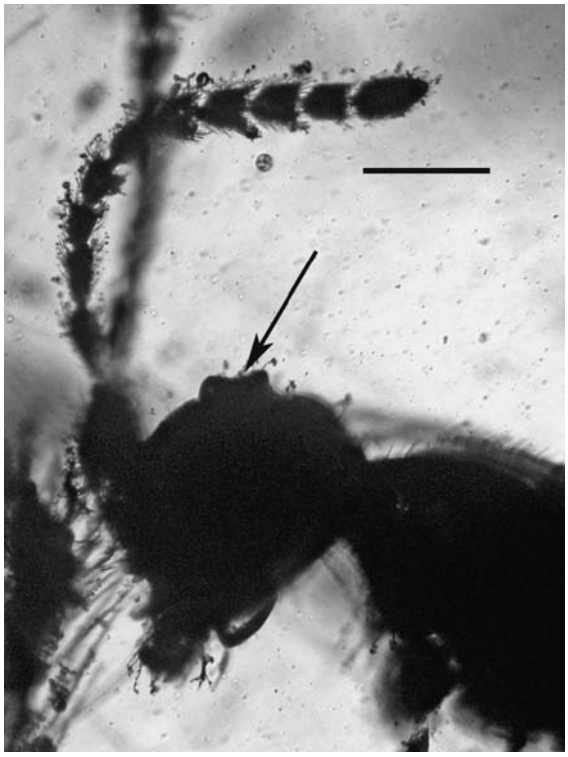

Figure 5. Head of *C. incassus* in Early Cretaceous Burmese amber showing antenna and ocellar tubercle (arrow) Bar = 127 μm.

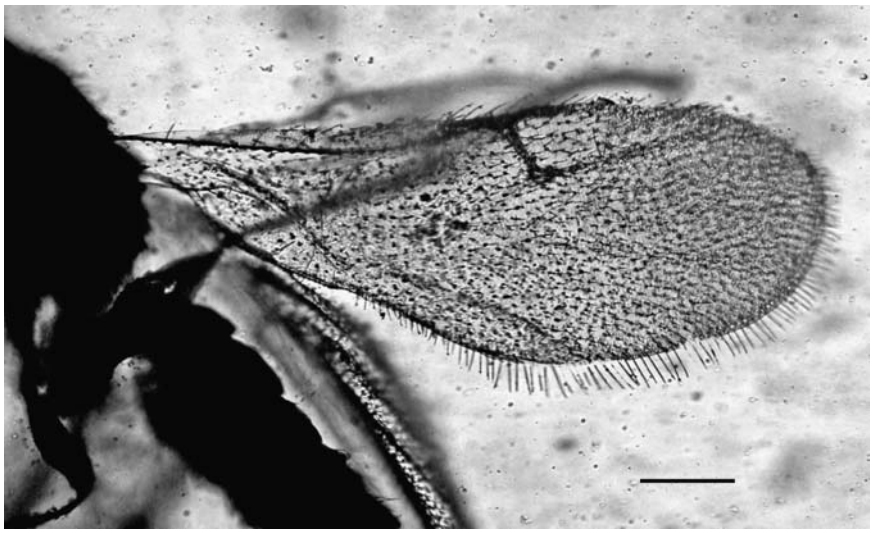

Figure 6. Forewing of *C. incassus* in Early Cretaceous Burmese amber. Bar = 114 μm.

than to median ocellus; mandibles bidentate, 53 μm long; antennae 12-segmented with feebly five-segmented clava; clava segments setose; scape elongate, more than five times longer than wide; flagellomeres 8–11 subequal, quadrate; terminal flagellomere pointed, longer than preceding flagellomeres; length antennomeres: scape, 126 μm; pedicel, 57 μm; 3rd antennomere, 86 μm; 4th antennomere, 57 μm; 5th antennomere, 46 μm; 6th antennomere, 52 μm; 7th antennomere, 52 μm; 8th antennomere, 57 μm; 9th antennomere, 57 μm; 10th antennomere, 52 μm;11th antennomere, 56 μm; 12th antennomere, 92 μm; elongate, thick STC with rounded ends ranging from 7 to 13 μm in length on ventral surface of claval antennomeres.

Thorax: Mesonotum minutely punctate; propodium medially armed with short spine; trochantellus absent; tibial spur formula 1-1-1; forewing length, 923 μm; wing membrane covered with thick short setae 18 μm in length directed towards wing apex; marginal cilia long, 14–60 μm in length; pigmented submarginal vein complete, tubular, reaching wing margin; postmarginal vein subequal to marginal vein and stigmal vein; uncus short with nebulose Rs vein extending from tip of uncus

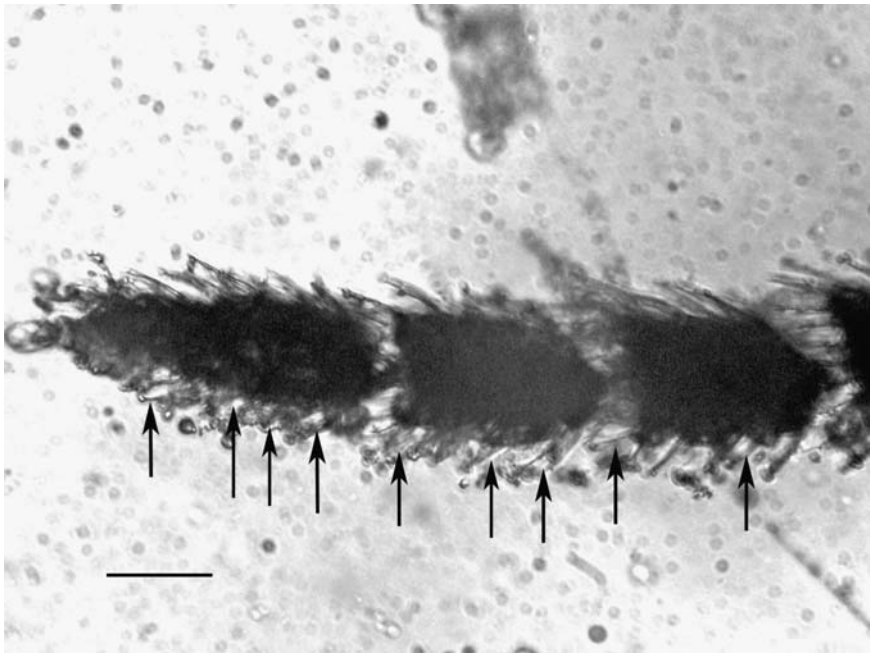

Figure 7. STC (arrows) on terminal three antennomeres of *C. incassus* in Early Cretaceous Burmese amber. Bar = 28 μm.

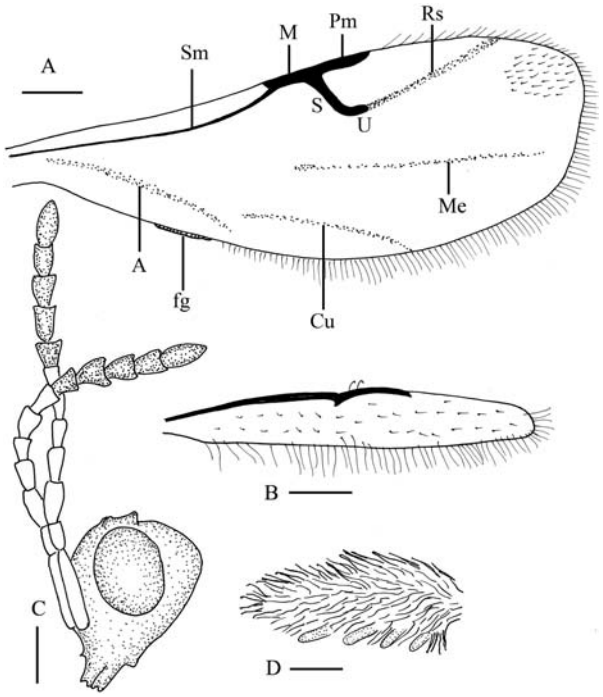

Figure 8. *C. incassus* in Early Cretaceous Burmese amber. (A) Forewing: A represents nebulose anal vein; Cu represents nebulose cubitus vein; fg represents frenal gutter; M represents marginal vein; Me represents nebulose medius vein; Pm represents postmarginal vein; Rs represents radial sector; S represents stigmal vein; Sm represents submarginal vein; U represents uncus. Only a few setae on wing membrane are shown. Bar = 95 μm. (B) Hind wing. Bar = 93 μm. (C) Head showing antennae with claval segments stippled. Bar = 90 μm. (D) Terminal antennomere showing four STC (stippled) on ventral surface. Bar = 22 μm.

straight to wing margin; anal, cubitus and medial nebulose veins present; only cubitus vein reaching wing margin; hind wing length, 580 μm; membrane with short, thick setae 14 μm in length; marginal cilia long, 36–54 μm in length; submarginal vein complete, reaching costal margin and continuing beyond paired frenal hooks.

Metasoma: Brown, shiny, setose, nine-segmented; length, 568 μm; width, 186 μm; 3.1 times as long as wide; laterotergites narrow, forming sharp submarginal ridge (lateral margin); T2 longest; length first six segments: 1, 105 μm; 2, 114 μm; 3, 81 μm; 4, 65 μm; 5, 73 μm; 6, 65 μm; tip (14 μm) of aedeagus exposed.

Type: Holotype deposited in the Buckley amber collection (accession No. E-2).

Type locality: Amber mine at the Noije Bum 2001 Summit site in the Hukawng Valley, south-west of Maingkhwan in Kachin State (26°20′N, 96°36′E) in Burma (Myanmar).

Etymology: Cassa is from the Latin ‘cassus’ for spider web.

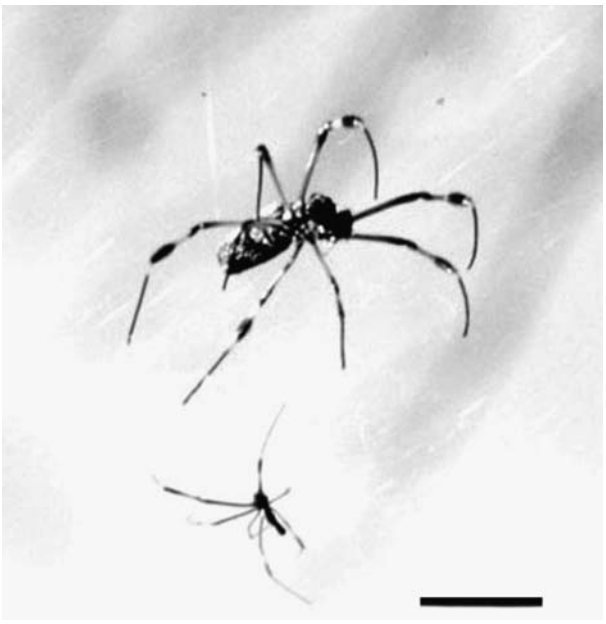

Figure 9. Smaller male (lower specimen) of the extant *Nephila clavipes* living in the web of the larger female (upper specimen). Photo taken by the author in Chiapas, Mexico. Bar = 13 mm.

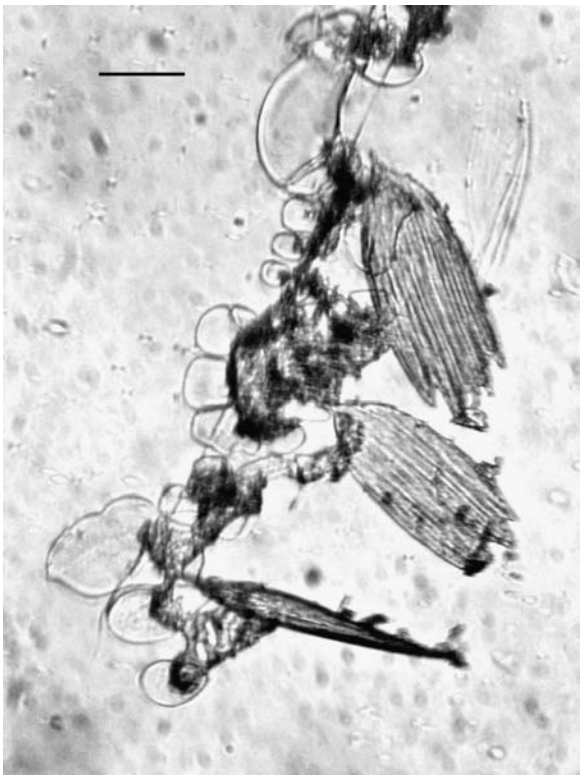

Figure 10. Scales attached to web of *G. burmanica* in Early Cretaceous Burmese amber. Note also large viscid droplets. Bar = 38 μm.

Comments: The fossil wasp falls into the scelionine section of the Platygastridae. It is not possible to assign the fossil to an extant or extinct genus based on the works of Masner (1976, 1980) and Masner et al. (2007). The presence of an ocellar tubercle (Figures 5, 8(C)), a five-segmented feeble club, short postmarginal vein and other wing characters (Figures 6, 8(A)) separates *Cascoscelio* from Tertiary members of platygastrids described from Baltic amber, which includes species in the genera *Aneurobaeus* Kieffer, 1912; *Archaeoscelio* Brues 1940; *Brachyscelio* Brues 1940; *Ceratobaeoides* Dodd, 1913; *Ceratoteleia* Kieffer, 1908; *Dissolcus* Ashmead, 1893; *Electroteleia* Brues 1940; *Gryon* Holiday, 1833; *Hadronotoides* Dodd, 1913; *Hoploteleia* Ashmead, 1893; *Microtelenomus* Dodd, 1913; *Proplatyscelio* Brues 1940; *Pseudobaeus* Perkeins, 1910; *Sembilanocera* Brues 1940; *Trachelopteron* Brues 1940 and *Uroteleia* Brues 1940. While the Baltic amber *Sparaison simplicifrons* Brues 1940 has a broad tubercle between the face and vertex that is somewhat similar to the ocellar tubercle on *Cascoscelio,* no ocelli are associated with the tubercle of *S. simplicifrons* and that species has a seven-jointed club, the postmarginal vein extends to the tip of the radial cell and the stigmal vein is curved (Cockerell 1909; Brues 1937, 1940; Masner et al. 2007).

Cobaloscelio Masner and Johnson, 2007 from Baltic amber has the mesopleuron and metapleuron completely fused, the frons with a well-developed median longitudinal carina, a seven-segmented clava, short marginal cilia on the forewing, a short submarginal vein remote from the costal margin, R_1 not reaching the wing margin and Rs as an arched nebulous vein (Masner et al. 2007). The Baltic amber *Chromoteleia theobaldi* Maneval (1938) has a seven-segmented clava, the mandibles are tri-dentate and the postmarginal vein is much longer than that of *Cascoscelio*.

The Mexican amber *Palaeogryon muesebecki* Masner (1969) is only 0.6 mm in length, antennomeres 3–6 are transverse and the club is only three segmented. The genus *Moravoscelio* (Nel and Prokop 2005) in Eocene Moravian amber has reduced antennal segments 3–6 and a completely different forewing venation than *Cascoscelio*. The genus *Galloscelio* Nel and Prokop (2005) in French Eocene Oise amber has a six-segmented club and the venation in both fore and hind wings differs significantly from that of *Cascoscelio*.

Three species of platygastrids were described from Upper Cretaceous Canadian amber (Brues 1937). Of these, *Baryconus fulleri* Brues 1937 has a six-segmented club, the 3rd tergite is much longer than T1 and T2 combined and the marginal vein is short, only half as long as the stigmal vein. *Baeomorpha dubitata* Brues 1937 has nine-segmented antennae inserted high on the face with a large compact club. *Proteroscelio antennalis* Brues 1937 has a flattened head and 14-segmented antennae. The Late Cretaceous *Cenomanoscelio pulcher* Schlüter (1978) from French amber has 11-segmented antennae and antennomeres 4–6 are reduced and transverse. Also this wasp has a large head with the eyes occupying most of the lateral surface. The Early Cretaceous Lebanese amber *Proteroscelio gravatus* Johnson, Musetti and Masner (2008) has a 14-segmented antenna. All of the above characters differ from those of *Cascoscelio*. The present authors could find no previous descriptions of platygastrids in Burmese amber.

Discussion

G. burmanica provides fossil evidence of sociality in spiders. The presence of a male and juvenile in the same web implies the presence of a female since extant male nephilids live in female webs (Figure 9) (Comstock 1948). Spider sociality involves the cohabiting of juveniles and adults in a common web and cooperating in web construction and/or the acquisition of food. The advantage of sociality is an extended web to cover more area and maximise prey acquisition. The adults and pre-adults show a tolerance towards each other and their common young (Buskirk 1981). The males (extant webs of *Nephila* often contain more than one male) attend the female and share her food (Vollrath 1980). The presence of the juvenile spider indicates that intraspecific aggression and cannibalism were maintained at a minimal level, which are characteristics of social spiders. Although sociality in extant spiders evolved in several independent lineages, there was no previous fossil record of this behaviour, making its evolution difficult to interpret (Buskirk 1981).

At least 15 unbroken strands of spider silk extend through the entire length of the amber piece (longest continuous silk strand = 12.7 mm) and these major strands (1.6–2.0 μm in diameter) are crisscrossed by numerous thinner strands (0.5–0.7 μm in diameter) (Figure 1). The adhesiveness of the web is not only demonstrated by the variously sized viscid droplets (4–25 μm in diameter) attached to the strands, but also by the captured insects and attached lepidopteran scales (102–122 μm in length and 45–50 μm in width) (Figure 10). Many of the viscid droplets are covered with aerial plankton (pollen, spores and dust particles), which can be explained if the fossil clade had habits similar to extant nephilids that do not rebuild their webs at frequent intervals, but only repair damaged parts (Nentwig 1985). The large viscid droplets noted on the fossil strands (Figure 10) occur on webs of extant *Nephilia* as well, in which the larger drops often can be detected with the unaided eye (Comstock 1948). The use of viscid droplets by spiders to entrap their prey extends back at least to the Early Cretaceous (Zschokke 2003).

Parasitoid wasps usually have some type of sensory organs on their terminal antennomeres to detect hosts or mates. In platygastrids, several types of sensilla occur on

the antennae of both males and females (Bin 1981; Cave and Gaylor 1987). It is difficult to determine how many sensilla are present on the claval antennomeres of *Cascoselio*. Only nine are clearly visible on the ventral surface of the terminal three antennomeres (Figure 7), with four on the terminal antennomere (Figures 7 and 8(D)). They are similar in size (7–13 μm) to the STC in males of *Telenomus reynoldsi* Gordh and Coker, 1973 (10–14 μm); however, those of the fossil are thicker and have more rounded rather than pointed ends.

Aside from *Cascoselio*, a second captured insect is a partially wrapped neuropteran (length of the remaining body, 2 mm) adjacent to the male spider (length, 3 mm) (Figure 1). *Cascoselio* is attached to the web by at least six strands of silk and three legs of the juvenile *G. burmanica* are in contact with the wasp (Figures 2 and 4). It would appear that *Cascoselio* was entrapped only seconds before the resin covered the web, which appears to have occurred just as the arachnid was preparing to feed on its prey. Studies on the extant *Nephila clavipes* (L.) that forms nests in shrubs and trees showed that over 9% of the total insect prey captured were parasitic Hymenoptera ranging between 1 and 2 mm in length (Nentwig 1985).

Aside from providing the first fossil preservation of an orb web together with its spider builder and captured prey, the present study demonstrates the existence of spider sociality in the Early Cretaceous.

Acknowledgements

We thank Pat Craig and Darrell Ubick for their assistance in designating the systematic position of *Geratonephila burmanica*, Lubomir Masner for determining the wasp as an undescribed genus, Samuel Zschokke for interpretation of the viscid droplets in the spider web and Art Boucot, Andy Moldenke and Roberta Poinar for comments on earlier drafts of the manuscript.

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Grizzly bear predation links the loss of native trout to the demography of migratory elk in Yellowstone

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Grizzly bear predation links the loss of native trout to the demography of migratory elk in Yellowstone

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The loss of aquatic subsidies such as spawning salmonids is known to threaten a number of terrestrial predators, but the effects on alternative prey species are poorly understood. At the heart of the Greater Yellowstone ecosystem, an invasion of lake trout has driven a dramatic decline of native cutthroat trout that migrate up the shallow tributaries of Yellowstone Lake to spawn each spring. We explore whether this decline has amplified the effect of a generalist consumer, the grizzly bear, on populations of migratory elk that summer inside Yellowstone National Park (YNP). Recent studies of bear diets and elk populations indicate that the decline in cutthroat trout has contributed to increased predation by grizzly bears on the calves of migratory elk. Additionally, a demographic model that incorporates the increase in predation suggests that the magnitude of this diet shift has been sufficient to reduce elk calf recruitment (4–16%) and population growth (2–11%). The disruption of this aquatic–terrestrial linkage could permanently alter native species interactions in YNP. Although many recent ecological changes in YNP have been attributed to the recovery of large carnivores—particularly wolves—our work highlights a growing role of human impacts on the foraging behaviour of grizzly bears.

1. Introduction

In many ecosystems, spawning salmonids provide subsidies to riparian and terrestrial food webs when predators consume them or move their carcasses to land [1,2]. The abundance of salmonids and other aquatic prey has been linked to the survival, fecundity and density of terrestrial consumers including spiders and lizards [3], passerine birds [4], coyotes (*Canis latrans*; [5]), wolves (*Canis lupus*; [6]) and brown or grizzly bears (*Ursus arctos*; [7]). However, much less is known about the indirect effects of these subsidies on alternative resources in the recipient, terrestrial community [4,8]. Such ecological interactions can have important conservation implications if the loss of a primary prey species results in disproportionate, but cryptic, impacts on alternative prey species that occur at lower abundance [9]. A recent, dramatic decline of cutthroat trout (*Oncorhynchus clarkii* bouvieri) in Yellowstone Lake, at the heart of Yellowstone National Park (YNP), has been associated with increased predation on elk (*Cervus elaphus*) calves by the omnivorous grizzly bear [10]. Here, we explore the potential influence of this diet shift on migratory elk that winter 40–100 km from Yellowstone Lake, far beyond the boundaries of YNP.

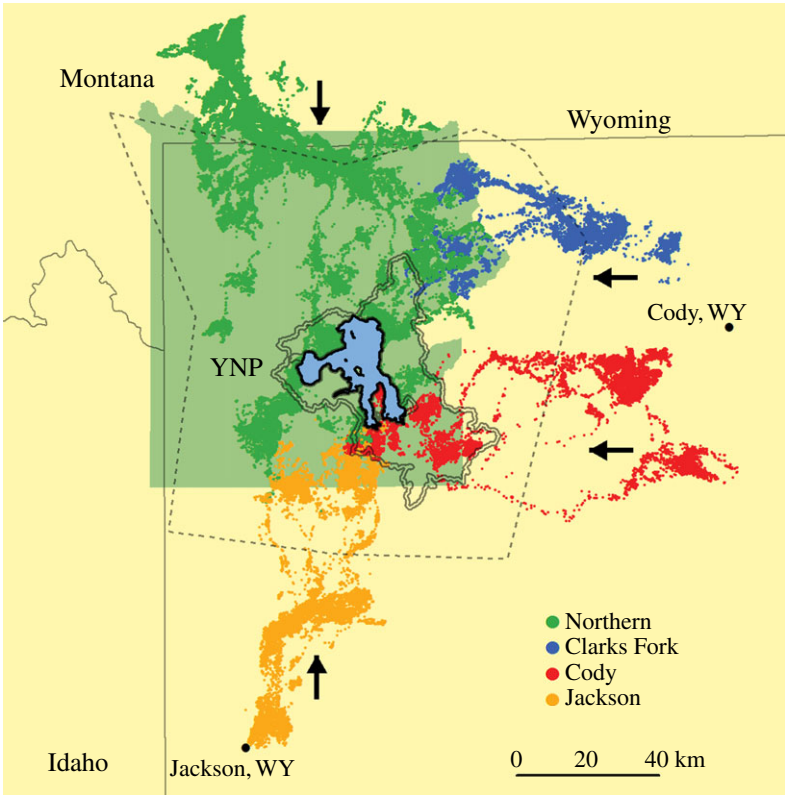

Figure 1. Individuals in four elk populations migrate each spring from outlying areas of the GYE to high-elevation summer ranges in and around the watershed of Yellowstone Lake. Here, the year-round movements of 5–10 individuals in each population are pooled to illustrate migratory movements, with a global positioning system fix rate of 1–12 locations per day. The double line delineates the Yellowstone Lake watershed; the dotted line, a polygon built from the aggregated year-round VHF locations of grizzly bears known to feed on cutthroat trout during the 1980s (adapted from Mattson & Reinhart [14]). Black arrows indicate the direction of migration from winter to summer ranges.

The Greater Yellowstone ecosystem (GYE) harbours one of the most diverse assemblages of large mammals in North America. The return of native large carnivores to YNP, including the reintroduction of wolves and recovery of grizzly bears, is widely thought to have restored ecosystem functioning [11,12]. Simultaneously, the introduction of a non-native aquatic predator, the lake trout (*Salvelinus namaycush*), has emerged as a major conservation problem for YNP [13]. Historically, Yellowstone Lake (figure 1) harboured an abundant population of cutthroat trout, but lake trout prey heavily on cutthroat trout [15] and have driven a decline of more than 90 per cent in their numbers [13]. Although cutthroat trout migrate up shallow tributary streams to spawn, and are exploited by many terrestrial predators, lake trout spawn on the lake bottom and are inaccessible to those predators [13,15]. The lake trout invasion is thought to have influenced the foraging of many birds and mammals [13,16,17], but its cascading ecological consequences are largely unknown.

Spawning cutthroat trout were an important prey species for a portion of the GYE's population of grizzly bears [14,18,19], which incorporate many vertebrates, invertebrates and plants into their diets [18,20]. We explore one consequence of this omnivory, an ecological linkage between the aquatic and terrestrial food webs of the GYE that arises from the spatial and temporal coincidence each spring of cutthroat trout spawning with elk migration. We hypothesize that an increase in the rate of grizzly predation on elk calves, caused by the lake trout invasion and cutthroat trout decline [10], has contributed to the declining productivity of migratory elk in the GYE (figure 2). Many elk that spend spring and summer in high-elevation habitats near Yellowstone Lake migrate 40–140 km to winter ranges outside of YNP—a

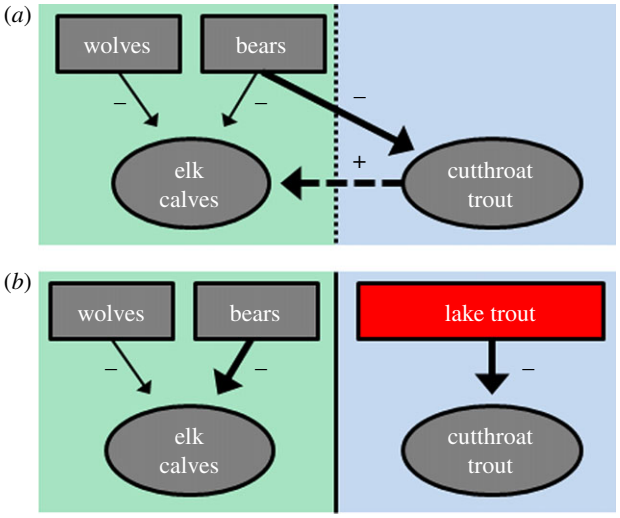

Figure 2. Focal food web interactions (*a*) before and (*b*) after the lake trout invasion in Yellowstone Lake. Predation by lake trout has driven a precipitous decline in the number of native cutthroat trout. Unlike cutthroat trout, which migrate up shallow streams to spawn, lake trout spawn on the lake bottom. Thus, the lake trout invasion has disrupted a major aquatic subsidy to terrestrial consumers, such as the grizzly bear.

behaviour that may transmit the consequences of the lake trout invasion far beyond park boundaries (figure 1).

We evaluate this hypothesis by first synthesizing historical and contemporary studies, including new data, that address three interrelated ecological patterns in and around the watershed of Yellowstone Lake: (i) elk migration and calving; (ii) decreased fishing activity by grizzly bears; and (iii) increasing rates of predation on elk calves by grizzly bears. Then, to

evaluate the potential strength of the linkage from lake trout invasion to elk migration, we incorporate observed shifts in grizzly bear diets into a model of elk demography to evaluate changes in elk calf recruitment and population growth. We also discuss several alternative hypotheses for our observations. Ultimately, while the growing abundance of large carnivores and a recent drought have also influenced calf recruitment of migratory elk [21], the role of a changing grizzly bear diet is of singular management concern because of its anthropogenic origin at the heart of the vast YNP wilderness.

2. Elk migration and calving in and around the watershed of Yellowstone Lake

Several thousand elk migrate each spring from outlying GYE winter ranges on mixed-use lands in Montana and Wyoming, up to wilderness summer ranges inside YNP. This includes individuals from four major populations, among them the well-studied northern Yellowstone herd [21–23]. Our synthesis of recent global positioning system (GPS) collar data and population surveys reveals that many of these elk migrate to access summer ranges in or near the watershed of Yellowstone Lake (figure 1). Thus, while this watershed comprises only approximately 30 per cent of YNP and approximately 3 per cent of the GYE, perturbations in and around Yellowstone Lake might disproportionately impact the ecosystem's migratory elk.

Yellowstone's spring elk migrations typically begin in mid-May [23], and are followed by the peak of elk calving around 1 June [24,25]. Most predation by bears on elk occurs in the three weeks after calving, when elk neonates are most vulnerable [24,25]. Variation in winter severity, spring snowmelt and vegetation green-up can cause the onset of elk migrations to vary by more than a month [23], which influences the spatial distribution of elk calving sites along a gradient in bear density that reaches its peak within YNP. Nevertheless, in a typical year, large numbers of elk calve in and around the watershed, whereas others arrive later with young neonates that vary in their vulnerability to predation.

We compiled a series of winter elk surveys conducted over the past two decades in the GYE (see the electronic supplementary material). They indicate that on winter ranges dominated by migratory elk, calf recruitment has been declining since the late 1990s (figure 3*e*), with calf–cow ratios reaching 0.1 to 0.2 for most of the past decade [21]. By contrast, the median winter calf–cow ratio between 1978 and 2006 across Wyoming's elk herds outside of the GYE was 0.41 [28]. Although these surveys suggest steady declines among migrants, they have limited value in determining the role of neonate mortality because they are conducted six months or more after calving, in areas where migrants often mix with residents. Thus, we conducted new aerial surveys on elk summer ranges in and around the Lake watershed (see the electronic supplementary material). These data suggest that the calf–cow ratios have declined to low levels by late summer (figure 3*e*). Most strikingly, segments of the northern Yellowstone herd that summer near Yellowstone Lake have been observed with calf–cow ratios below 0.1 in July and August [29]. Such low calf numbers, relatively soon after calving, suggest a combination of low pregnancy [21], low birth weights [27] and/or high rates of predation [24]. However, pregnancy rates in the northern Yellowstone herd have been more than 80 per cent in recent years [29,30], and

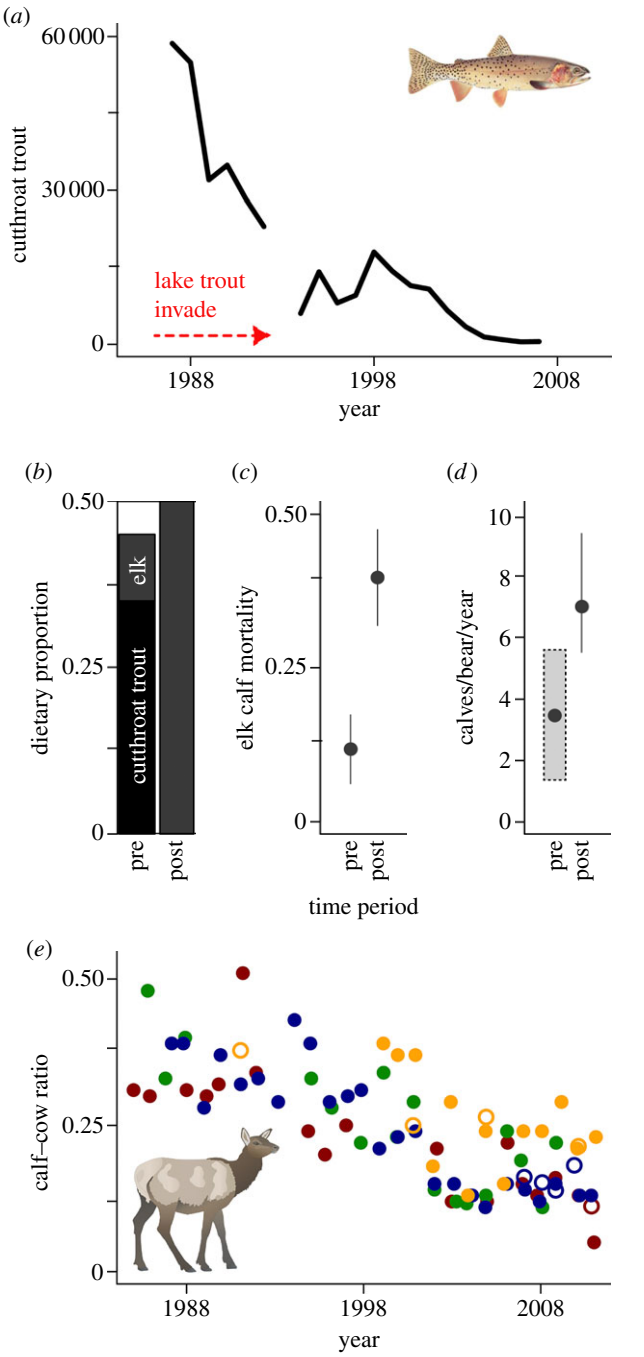

Figure 3. (*a*) Since the late 1980s, the number of spawning cutthroat trout counted each spring at Clear Creek (YNP's primary long-term monitoring site) has declined. We broadly define the 'pre-decline period' as before 1998, and the 'post-decline period' as after 1998. (*b*) In studies conducted during the post-decline period, the proportion of trout in the grizzly bear diet (black) at peak calving/spawning time has decreased, whereas the proportion of ungulate tissue (grey) has increased (estimates from Fortin *et al.* [10], Mattson & Reinhart [14] and Mattson [26]). (*c*) The proportion of elk calf mortality (±95% confidence interval (CI)) attributed to bear predation (primarily grizzly bears; [24,27]) and (*d*) the *per capita* rate of predation by grizzly bears on elk calves has increased over the same time period [10,26]. In (*d*), the shaded box indicates an estimated range for the number of ungulates killed per bear per year, and the black dot indicates its median value, which we conservatively assumed to represent elk calves only and used in our demographic models. (*e*) The winter calf–cow ratios of migratory elk from four GYE populations (closed circles) have declined steadily over the same period, and comparable summer (August–September) surveys (open circles) suggest that calf losses occur largely before summer's end. The colours in panel (*e*) correspond with those shown in figure 1. Instances where a population's summer ratio exceeds its winter ratio are probably attributable to subpopulation mixing on winter range.

recent study of calf mortality did not find any correlation between birth weight and the risk of mortality [24]. These patterns suggest that summer predation has contributed to low calf–cow ratios in migratory populations [21,24].

3. Declining grizzly fishing activity on cutthroat spawning streams

Bears are known to feed on spawning salmonids in many ecosystems [7]. Cutthroat trout have long been considered an important food for a portion of YNP's grizzly bear population [14,31], providing concentrated fat and protein at a critical time of the year when bears are recovering from hibernation [18,19]. Approximately half of Yellowstone Lake's 124 tributary streams were historically used by cutthroat trout, which spawn between mid-May and early August [14,19]. Early studies found that grizzly bears fished on most active spawning streams in most years [14]. One recent (1997–2000) estimate indicated that 68 individual grizzly bears, or 14–21% of the GYE population, visited and may have fished the tributaries of Yellowstone Lake from May to July [19]. Earlier studies indicated that cutthroat trout comprised the majority of these grizzly bears' diet during the spawning period [14].

Since the late 1980s, the number of cutthroat trout in Yellowstone Lake has declined substantially. On some key tributaries, the number of spawning trout has declined by more than 90 per cent since 1990 (figure 3*a*) [13]. Over this same period, the number of bear scats and tracks, partially consumed trout remains and grizzly bear visits per week have decreased along active spawning streams [13,19]. By 1997–2000, the estimated proportion of cutthroat trout in grizzly bear diets had dropped by as much as 90 per cent [32]. By 2007–2009, trout consumption had declined another 72 per cent, such that trout appeared only rarely in the diet (figure 3*b*; [10]). The loss of cutthroat trout has led many biologists to speculate that grizzly bears would seek alternative foods, and potentially suffer demographic consequences [13,19,33].

4. Increasing grizzly predation on elk neonates

Several lines of evidence suggest that newborn elk are an alternative prey for grizzly bears faced with declining availability of spawning cutthroat trout. Bears are adept predators of neonatal ungulates in many areas of North America [34], including the GYE [24,26,27]. Trout spawning and elk migration overlap both spatially and temporally [19,24], and the tissues of spawning trout and elk calves are similar in their nutritional value [35]. Further, in comparison with other North American landscapes occupied by grizzly bears, the GYE has less abundant nutritious plant matter [7] including relatively poor berry production [18]— leaving bears with comparatively few high-quality alternatives to animal tissue.

In the early and middle twentieth century, naturalists anecdotally described grizzly bears consuming trout commonly, but elk calves only occasionally [31,36]. More recently, in the years spanning the cutthroat decline, a growing proportion of elk calf mortality in YNP has been attributed to bear predation. In the late 1980s, grizzly and black bears (*Ursus americanus*) killed an estimated 12 per cent of the elk calves in northern Yellowstone annually [27]. By the mid-2000s, bears were estimated to kill 41 per cent of calves (figure 3*c*) [24]. In both cases, most of this

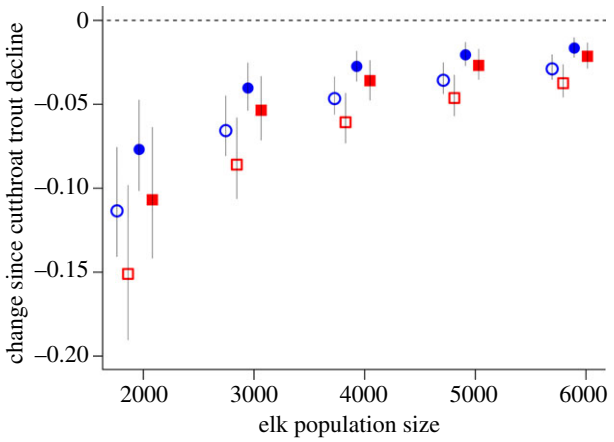

Figure 4. Predicted changes in elk calf–cow ratios (open symbols) and population growth rate (λ, closed symbols) owing to the cutthroat trout decline, using estimates based on estimated kill rates (red squares) and biomass replacement of trout with elk calves (blue circles). Elk were modelled over a range of population sizes owing to uncertainty in the number of elk that summer in and around the Yellowstone Lake watershed. For reference, a composite sum taken from summer surveys conducted in August 2008, 2010 and 2011 (conducted by the Wyoming Game and Fish Department and Montana Fish, Wildlife and Parks) suggests a minimum population of 2383 adult females. All values are presented as means $\pm$95% CI.

predation was attributed to grizzly bears. To date, researchers have assumed that these increases in bear predation reflected an increase in bear numbers [21,24], rather than dietary shifts. However, a comparison of historical and contemporary grizzly diet studies suggests that the *per capita* rate of elk calf predation by grizzly bears increased over the same period. In the late 1980s, the first large-scale study of the use of ungulates by grizzly bears estimated that an individual grizzly killed 1.4–5.8 ungulates per year, 13 per cent of which were elk calves [26]. By contrast, more recent studies have estimated that an individual grizzly on Yellowstone's northern range kills 19 calves per year [24]—and within the Yellowstone Lake watershed, seven calves during the month of June (figure 3*d*; [10]). In parallel with these increases, in the late 1980s ungulate tissue was estimated to comprise 5 per cent of the grizzly diet at peak calving time (figure 4; [14,18,26])—but more recently, above 50 per cent [10]. Although the earlier study was based on VHF telemetry [26] and might have detected fewer calf predation events, a correction factor was applied based on observations of the amount of time grizzly bears spent at carcases of varying size (see the electronic supplementary material for additional discussion).

This apparent historical-to-contemporary shift in bear foraging behaviour has been strongly corroborated by a comprehensive study of bear diets and behaviour conducted in the Yellowstone Lake watershed from 2007 to 2009 [10], which coupled stable isotope and mercury analyses of shed hair with GPS-based feeding site visits and faecal screening. This recent study found that while male grizzly bears (formerly the primary beneficiary of cutthroat trout) now consume one-third less meat as they did 30 years earlier, female grizzly bears consume the same amount of meat [10,32]. In concert with observations of frequent elk calf predation and large amounts of ungulate tissue in many faecal samples, these findings indicate that female grizzly bears have replaced the lost cutthroat trout biomass with that of elk neonates. This work has also found that the number of

grizzly bears visiting historical spawning streams declined by 31 per cent [10] following the decline of cutthroat trout, suggesting that the effect of the cutthroat trout decline on grizzly bear behaviour could extend over a larger geographical area. Indeed, grizzly bears range widely; in the GYE, their distribution varies with the availability of human refuse, whitebark pine (*Pinus albicaulis*) seeds and ungulate 'gutpiles' left by hunters [37,38], and grizzly bears from a large area were historically thought to concentrate along tributaries of Yellowstone Lake during the spawning season (figure 1; [14]). Because very few (if any) female elk reside year-round in or near the watershed of Yellowstone Lake (P. J. White, D. E. McWhirter and D. G. Brimeyer 2012, personal communication), the influence of this diet shift can only be apportioned among migratory elk, and could impact their demography [10].

5. Evaluating the potential demographic effect of the cutthroat trout decline on migratory elk

To evaluate the hypothesis that grizzly bear diet-switching has influenced migratory elk demography, we first calculated the number of elk calves that grizzly bears might newly consume as a consequence of diet shifts, then used an age-structured elk population model to explore how this additional calf predation could influence elk calf recruitment and population growth. We assumed that the 68 bears estimated to fish along the tributaries of Yellowstone Lake in the late 1990s [19] replaced the trout biomass in their diet with equivalent elk calf biomass [10], and that the number of grizzly bears inside YNP did not change during the cutthroat decline (see fig. 5 in Schwartz *et al.* [39]). One of our most important assumptions was that calf mortality from bear predation is additive (supported by Griffin *et al.* [25]; see also [24,34]). Bear predation is thought to be additive because bears specialize on killing neonates before individual heterogeneity (e.g. body condition) begins to strongly mediate vulnerability [24,25].

We first calculated the number of elk calves that would be required to replace the trout biomass lost from the diet of grizzly bears. Prior to the cutthroat trout decline, 44 grizzly bears were estimated to eat 20 578 spawning trout, weighing 468 g each, or a total of 9630 kg per year [40]. This study probably overestimated cutthroat trout consumption [10,32] because of its assumption that scats sampled along streams [14] represented the diets of grizzly bears foraging further afield in the Yellowstone Lake watershed during the spawning period. We addressed this issue by using the product of the historical estimate of the proportion of trout in the diet (0.9; [14,40]) and the proportion of VHF locations of probable trout-eating grizzly bears that fell near (within 2 km) tributary streams during the spawning period (0.38; [14]). This resulted in a greatly revised estimate of 7820 trout (3659 kg) consumed per year. Using the more recent estimate of 68 individuals fishing in the Yellowstone Lake watershed between 1997 and 2000 when trout were still relatively abundant [19], the local population would be estimated to have consumed 5656 kg of trout per year. By contrast, after the bulk of the cutthroat trout decline (2007–2009), grizzly bears were estimated to eat only 302 spawning trout (314 kg) per year [10]. Assuming a 1 : 1 nutritional equivalency of trout and elk biomass (probably a conservative assumption owing to the high digestibility of trout [14] and potentially higher metabolic costs of hunting more sparsely distributed elk calves) and a calf weight of 18 kg each when killed by grizzly bears [26], the resulting 5342 kg loss of trout biomass would be replaced with approximately 297 elk calves.

We calculated a second, independent estimate of change in grizzly bear predation rates on elk calves using predation rates that were estimated before and after the cutthroat trout decline. Recognizing the inherent limitations of historical studies that used VHF telemetry to locate kills, we used the median (3.6) of the estimated pre-decline kill rate of 1.4–5.8 ungulates per grizzly bear per year [26] and assumed this kill rate was for *elk calves only* (a conservative assumption that reduces the predicted changes in elk calf–cow ratios and population growth). Thus, 68 individuals in the Yellowstone Lake watershed would have killed 245 elk calves annually. In the past decade in the Yellowstone Lake watershed, the same number of grizzly bears are estimated to kill 476 calves annually (seven calves per year, 10), for an estimated increase of 231 calves. Notably, this estimate broadly agrees with our above estimate, based on trout biomass replacement (297 calves).

To explore the potential impact of these changes on elk populations, we incorporated both sets of the above calculations into an age-structured elk population model (see the electronic supplementary material). Because the number of elk that mix in and around the Yellowstone Lake watershed has not been estimated and the population size may vary with annual migration timing, we predicted change in the rates of recruitment and population growth (λ) across a range of population sizes exposed to grizzly bear predation. Ultimately, our predictions were primarily determined by two inputs: (i) the estimated change in the number of calves being killed by grizzly bears and (ii) the overall size of the elk population. For reference, we note that a composite sum taken from surveys within three distinct areas of the Yellowstone Lake watershed in August 2008, 2010 and 2011 (conducted by the Wyoming Game and Fish Department and Montana Fish, Wildlife and Parks) suggests a minimum population of 2383 adult females by late summer.

Our simulations predicted an influence of grizzly bear diet-switching on elk calf recruitment and population growth rates across a wide range of potential population sizes (figure 4). Although the magnitude of the predicted changes depends both on the increase in calf mortality and the total population size, all combinations of estimates resulted in declines of both calf recruitment (0.04–0.16) and population growth (0.02–0.11). An explicit accounting of estimated changes in bear predation rates in our models indicated that shifts in bear foraging behaviour—an indirect consequence of lake trout invasion—are capable of creating meaningful changes in the population dynamics of migratory elk.

6. Alternative explanations

Our inferences draw on a large body of research conducted by biologists working independently, across multiple taxa, over several decades. The patterns we describe—the coincidence of cutthroat trout decline, grizzly diet shifts from trout to elk calves and the declining recruitment of migratory elk—are consistent with an emergent link between lake trout invasion and elk migration in the GYE. However, as is so often the case with 'natural experiments', it is challenging to determine cause and effect when evaluating food web changes spanning several decades in landscapes so vast as the GYE. Thus, we discuss several alternative explanations

for our observations, and explain why we suspect they do not oppose our findings.

Although predation by non-native lake trout is widely considered the leading cause of the cutthroat trout decline [13,41], at least two other factors play a role. An unusually severe, long-term drought reduced the flow levels of some tributary streams for much of the past decade, probably reducing cutthroat trout recruitment to the lake [13]. Additionally, the parasite *Myxobolus cerebralis*, which causes neurological damage (i.e. whirling disease), reduces the survival of juvenile cutthroat trout in some areas of Yellowstone Lake [13]. Whirling disease was introduced by humans [13], and a number of studies have linked recent drying and warming trends in the region to anthropogenic climate change [42–44]. Thus, regardless of the relative importance of lake trout predation versus secondary factors, the decline of native cutthroat trout is considered by many observers to be largely a consequence of human actions.

Although there is substantial evidence of changes in grizzly bear diets [10], recent increases in bear predation on elk calves are also probably a function of increasing grizzly bear numbers. In recent decades, the numbers and distribution of grizzly bears have grown in the GYE. However, this growth appears to have occurred primarily outside the core areas of YNP. From 1983 to 2002, the number of females with cubs, a key indicator of grizzly population productivity, did not increase inside YNP (see fig. 5 in Schwartz *et al*. [39]). This pattern suggests that grizzly bear habitat was saturated inside YNP [39]. If the proportion of elk calf mortality attributed to grizzly bears inside YNP increased more than threefold (cf. [24,27]) during a period when grizzly bear numbers did not increase, then it is logical that the *per capita* rate of predation increased (cf. [10,26]). However, it is important to note that in years of harsh winters, deep snow and late migration, more elk tend to calve in outlying areas of the GYE [23] where grizzly bears have been expanding and growing in numbers [39]. For these reasons, we suggest that the combination of more grizzly bears *outside* YNP (owing to their recovery) and changing grizzly bear diets *inside* YNP (owing to the decline of cutthroat trout) acts synergistically to reduce the calf recruitment of migratory elk.

In addition to predation by grizzly bears, predation by wolves and other predators [24] and low elk pregnancy rates in some areas [21] probably influence the calf recruitment of migratory elk. However, grizzly bears far outpace wolves and other predators as a cause of summer elk calf mortality [24,25], and reductions in pregnancy do not appear large enough to explain the decreases in summer calf–cow ratios that have recently been observed [21,24]. Wolf predation did not appear powerful enough to cause the pronounced decline of northern Yellowstone elk following wolf reintroduction [45]—and although human hunting probably played an important role, hunters tend to select adult elk, not calves. It is possible that other recent ecological and behavioural changes that are unrelated to the cutthroat decline have contributed to increasing rates of grizzly predation on elk calves. Several other key grizzly foods have declined in recent years, namely winter-killed ungulate carcases owing to predation and scavenging by reintroduced wolves, and whitebark pine seeds, owing to beetle (*Dendroctonus ponderosae*) and invasive fungal (*Cronartium ribicola*) infestations. Although we cannot rule out effects of these latter changes, we expect that their consequences have not been as dramatic as the loss of a diet item (i.e. cutthroat trout) that coincides both spatially and seasonally with the calving of many migratory elk.

7. Discussion

Recent changes in the productivity and abundance of migratory elk in the GYE are widely viewed as a consequence of recovering numbers of large carnivores, but new evidence suggests that the decline of native cutthroat trout has caused omnivorous grizzly bears to kill more elk calves in some areas of YNP. Predation by non-native lake trout has dramatically reduced the population of cutthroat trout that once provided critical nutrition to grizzly bears foraging at the core of the GYE, leaving bears to find alternative sources of fat and protein each spring. Historical and contemporary studies of grizzly bear diets and behaviour indicate that individuals in and around the watershed of Yellowstone Lake—an area which comprises 30 per cent of YNP—have made up for the loss of cutthroat trout by consuming elk calves at a higher rate (figure 3). This diet switch is consistent with summer elk surveys that reveal low calf numbers among the migratory populations that summer in and around the Yellowstone Lake watershed (figure 3*e*).

Our synthesis provides considerable support for an emergent link between lake trout invasion and the demography of migratory elk, but less clear is the magnitude of this effect. Demographic simulations suggest the effect has been large enough to contribute to meaningful reductions in the calf recruitment (4–16%) and growth rates (2–11%) of migratory elk populations (figure 4). These findings are consistent with the prediction from theory of subsidy influences in ecosystems that a consumer which aggregates to an ephemeral subsidy (i.e. spawning cutthroat trout), yet reproduces slowly (i.e. grizzly bears), will have relatively small effects on alternative resources (i.e. elk calves) in the recipient community. In the case we describe, however, this 'protective' effect of cutthroat trout on elk calves has been removed. While the growing abundance of large carnivores and a severe drought have probably played important roles in declining elk calf recruitment [21], we suggest that the contribution of changing grizzly bear diets to these declines is uniquely important to research and management because it represents a novel, human influence operating cryptically within core protected areas of YNP.

Our findings have important implications for ecosystem management and the conservation of aquatic–terrestrial linkages. Aquatic and terrestrial food webs have long been conceptualized as distinct ecosystem components [46]. This approach has been challenged by a growing recognition of strong cross-system subsidies and aquatic–terrestrial linkages [3,8], as in the case of spawning salmonids that subsidize upland riparian and terrestrial food webs in coastal North America [2]. Far inland, in the central watershed of YNP, a similar link appears to have been broken when the invasion of lake trout interrupted a crucial energy transfer from aquatic habitats, in the form of cutthroat trout biomass, to the terrestrial food web, via the foraging of grizzly bears (figure 2). Our work suggests that the probable consequences of lake trout invasion reach beyond the demography of cutthroat trout consumers [17], including grizzly bears [10], to that of such alternative prey as migratory elk that winter as far as 140 km away [23] in outlying areas of the GYE. Given that the grizzly bear is one of 28 mammals and birds that were thought to depend

on spawning cutthroat trout [16,17], the broader ecological consequences of lake trout invasion are potentially tremendous. It remains unclear whether historic levels of cutthroat trout spawning in Yellowstone Lake tributaries can be restored, and the ecosystem consequences of breaking this aquatic–terrestrial link reversed. Fisheries biologists and managers in YNP have worked intensively for more than a decade to suppress lake trout numbers via netting and removal from Yellowstone Lake [13,41]. In recent years, the success of this programme has increased through technological improvements and increases in the spatial and temporal targeting of high densities and sensitive age classes of lake trout [41]. Our findings underscore the broad ecological importance of these efforts, the urgency of identifying new methods to suppress lake trout and the value of preventing such invasions elsewhere.

The indirect interaction of lake trout and migratory elk that we describe has implications for the interpretation, conservation and management of large mammal interactions in the GYE. Wolves have been the focus of widely popularized accounts of YNP's trophic interactions [47], perhaps partly because they were controversially reintroduced, remain active year-round and conspicuously hunt elk. Relatedly, it is often assumed that the ecological effects of recovering large carnivores herald a return to a historical condition of the GYE, providing evidence of conservation success [11,12]. However, our work suggests that important effects of human disturbance and grizzly bear predation on migratory elk are being overlooked. Globally, declines of migratory ungulates are a subject of conservation concern [48,49].

Our findings are also relevant to the wolf management plans of Idaho, Montana and Wyoming, which generally allow the flexibility to increase wolf harvests in areas of declining elk productivity and abundance. Some of the steepest elk recruitment declines in these states have occurred in the GYE, coincident with wolf reintroduction. However, complex patterns of 40–140 km elk migrations that are unique to the GYE, compounded by high rates of bear predation inside YNP's boundaries, suggest that elk calf recruitment may not be as sensitive to wolf removal on some outlying winter ranges as to the number of grizzly bears and the availability of alternative grizzly bear foods on elk summer ranges in and around YNP. As wildlife managers seek to determine whether specific interventions are likely to ameliorate declines in elk calf recruitment, they may benefit from cooperative study and monitoring of migratory herds including the timing of elk calf losses (e.g. conducting more routine summer surveys), as well as elk pregnancy and cause-specific elk calf mortality.

Wildlife biologists and managers have long recognized the importance of monitoring and securing key grizzly bear foods in the GYE [18,39]. While our findings highlight the resiliency of omnivorous grizzly bears to a changing environment [10], they also highlight the grizzly bear's growing dependency on a reduced number of high-quality foods. Our synthesis and modelling did not incorporate the declining availability of whitebark pine seeds, but the foraging options of grizzly bears may become increasingly limited as stands of whitebark pine decline throughout the GYE [20]. Future research on the nature and extent of grizzly bear diet-switching in response to changing food availability will be critical to our understanding of Yellowstone's large mammal interactions—particularly those involving the primary prey and closest competitors of grizzly bears.

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Invasive Harlequin Ladybird Carries Biological Weapons Against Native Competitors

Andreas Vilcinskas *et al.*

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Invasive Harlequin Ladybird Carries Biological Weapons Against Native Competitors

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Invasive species that proliferate after colonizing new habitats have a negative environmental and economic impact. The reason why some species become successful invaders, whereas others, even closely related species, remain noninvasive is often unclear. The harlequin ladybird *Harmonia axyridis*, introduced for biological pest control, has become an invader that is outcompeting indigenous ladybird species in many countries. Here, we show that *Harmonia* carries abundant spores of obligate parasitic microsporidia closely related to *Nosema thompsoni*. These microsporidia, while not harming the carrier *Harmonia*, are lethal pathogens for the native ladybird *Coccinella septempunctata*. We propose that intraguild predation, representing a major selective force among competing ladybird species, causes the infection and ultimate death of native ladybirds when they feed on microsporidia-contaminated *Harmonia* eggs or larvae.

Human activities, particularly international trade, promote the spread of invasive species that cause extensive economic losses and negatively affect native species. Several factors can play a role in the invasive success of such species, including the lack of predators, short generation times, and the ability to disperse rapidly and adapt easily to new habitats (*1*, *2*). However, the principles that allow some species to become successful invaders, whereas most (even if closely related) do not, remain poorly understood (*3*). Invaders can be released from native, coevolved pathogens, but they face other pathogens in their new environments, suggesting that the ability to mount strong antimicrobial defenses may promote invasive success (*4*). Yet enhanced immunity can be costly and, therefore, can be traded off against other traits such as growth and reproduction (*5*, *6*). We used the harlequin ladybird *Harmonia axyridis* (a native species in central Asia) as a model to explore the potential role of immunity in invasion biology. This species has been introduced into many countries as a biological control agent against aphids and other insect pests but is now causing severe problems because it successfully outcompetes native ladybird species in many areas (*7*).

We recently showed that, in contrast to native ladybird species in Europe, the *Harmonia* hemolymph contains strong and constitutive antibacterial activity throughout development. We attributed this activity to harmonine, a secondary metabolite that accumulates to high levels in the hemolymph. The broad-spectrum activity of this alkaloid compound is demonstrated by its ability to inhibit even human pathogens such as *Mycobacterium tuberculosis* and *Plasmodium falciparum*, making it a promising lead for the development of new anti-infective drugs (*8*). Constitutive harmonine activity may help *Harmonia* to deal with pathogens encountered in new habitats, whereas native ladybirds are more susceptible to infection (*9*).

Independently, harmonine has been proposed as a chemical defense compound protecting *Harmonia* eggs and larvae from predation by native ladybird species (*10*). Kajita *et al*. reported that

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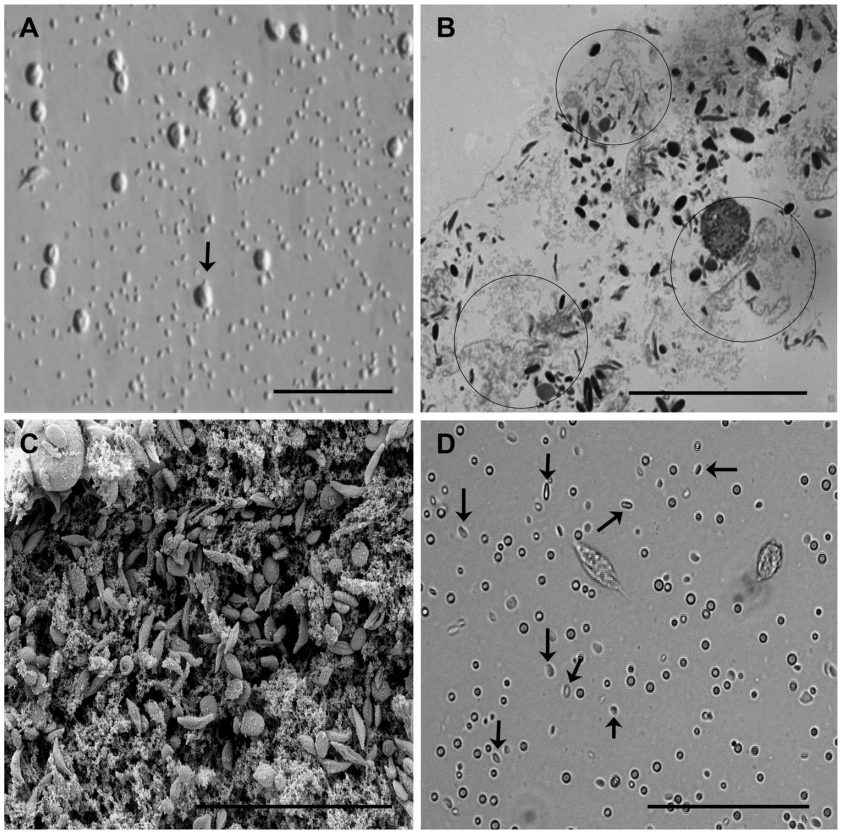

Fig. 1. Microscopy studies of microsporidia. (**A**) Light microscopy image showing the high concentration of microsporidia (small objects) between the larger hemocytes (arrow) in *Harmonia* hemolymph. Scale bar, 50 μm. (**B**) Semithin sections of hemolymph tissue confirm the presence of microsporidia, among which some exhibit extruded polar tubes (circles). Extrusion of polar tubes occurred during fixation. Scale bar, 20 μm. (**C**) Scanning electron microscopy image of microsporidia in the *Harmonia* hemolymph, showing the high load of spores. Scale bar, 20 μm. (**D**) Light microscopy image showing microsporidia (arrows) between yeastlike cells and larger hemocytes in dying *Coccinella* beetles 7 days postinoculation. Scale bar, 20 μm.

the ingestion of *Harmonia* eggs by native *Coccinella septempunctata* beetles caused mortality, but the reciprocal situation was nonlethal. In agreement with our previous report (*8*), they detected high concentrations of harmonine in *Harmonia* eggs and concluded that this compound protects the invasive ladybird from intraguild predation (*10*), which is a major selective force among competing ladybird species (*11*).

Here, we report that the injection of *Harmonia* hemolymph—but not synthetic harmonine alone, even in high concentrations (fig. S1)—can kill *Coccinella* beetles, making it unlikely that the mortality caused by feeding on *Harmonia* eggs is caused by the presence of harmonine. It is therefore apparent that the mortality in *Coccinella* beetles is caused by another component found in the hemolymph of *Harmonia*.

Light microscopy revealed the presence of abundant microsporidia among the hemocytes in *Harmonia* hemolymph (Fig. 1A) (see supplementary materials and methods). Microsporidia are obligate parasites that replicate within eukaryotic cells after penetrating the plasma membrane with an extruded polar tube. Semithin sections of hemolymph tissue confirmed the presence of microsporidia, among which some exhibited extruded polar tubes (Fig. 1B). We used scanning electron microscopy to document the high abundance of microsporidia in the hemolymph of *Harmonia* (Fig. 1C). Despite the abundance of spores, we did not find any *Harmonia* beetles that were killed by the parasites in our rearing. The low physiological activity of the microsporidia is further supported by the absence of microsporidial gene expression when analyzing the transcriptome of *Harmonia* eggs and beetles (*12*). These data suggest that the microsporidia are present in a physiologically inactive spore stage and do not harm their host, perhaps because it has acquired tolerance or resistance.

We identified the microsporidia by amplifying the small-subunit ribosomal RNA (rRNA) genes using a variety of previously described primer sets (*13*), resulting in the specific amplification of the partial microsporidial 16*S* rRNA gene. The determined sequence identity (≥99%) placed the *Harmonia*-associated microsporidia within the *Nosema*/*Vairimorpha* clade, with *Nosema thomsoni* as the closest relative (*14*). This assay confirmed the presence of *Nosema*-like microsporidia in all *Harmonia* beetles of all populations sampled, as well as in eggs and larvae, suggesting vertical transmission. All attempts to coculture the microsporidia with different insect cell lines (Sf9, Sf21, *Drosophila* S2, High Five) failed.

Given that (i) *Coccinella* beetles are killed by feeding on *Harmonia* eggs and larvae, but the reciprocal situation is not lethal (*10*), and (ii) harmonine does not affect *Coccinella* beetles, even at high concentrations, we propose that native ladybird species may be lethally infected by the microsporidia carried by *Harmonia* when they feed on its eggs and larvae. To test this hypothesis, we collected hemolymph samples from *Harmonia* and isolated the microsporidia by repeated centrifugation and washing steps. We divided the purified microsporidia into two portions, one of which was heat-inactivated and used for control injections.

All *Coccinella* beetles injected with live microsporidia isolated from *Harmonia* died within 2 weeks (Fig. 2), whereas the majority of control beetles injected with either the heat-inactivated microsporidia or the buffer alone survived. Control injections with cell-free hemolymph samples from *Harmonia* lacking microsporidia and hemocytes did not result in enhanced mortality (fig. S4). This observation and the analysis of injected samples by SDS–polyacrylamide gel electrophoresis (fig. S2) and mass spectrometry ruled out the possibility that the mortality was caused by thermolabile toxins in the hemolymph of *Harmonia*. Further, we determined the presence of microsporidia in dying *Coccinella*, but not in control beetles (Fig. 1D and fig. S3). We concluded that the microsporidia carried by *Harmonia* were lethal but required some time to infect and replicate within *Coccinella*.

The high abundance of tolerated microsporidia in *Harmonia* hemolymph and their ability to kill *Coccinella* beetles support our hypothesis that these parasites contribute to the dominance of *Harmonia* over native species. Obviously, native ladybirds such as *Coccinella* do not share with *Harmonia* the ability to suppress microsporidial replication. It remains to be seen whether or not harmonine (*8*) and/or the tremendous spectrum of antimicrobial peptides discovered in *Harmonia* (*12*) contribute to its tolerance or resistance against microsporidia. Our data also provide a candidate mechanism to explain why the decline in native ladybird numbers is associated with intraguild predation (*11*). The presence of microsporidia in *Harmonia* may function like a biological weapon, in accordance with the novel weapons theory (*15*).

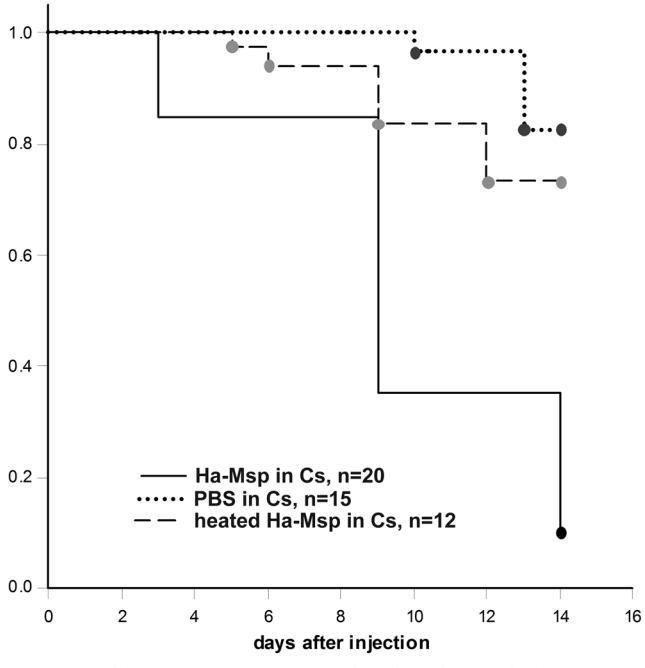

Fig. 2. Survival after injection. *Coccinella* beetle survival rate (*y* axis) analysis after transfer of microsporidia isolated from the hemolymph of *Harmonia* (Ha-Msp in Cs), calculated by Kaplan Meier survival analysis log-rank test. *Coccinella* beetles were injected with living microsporidia (solid line). Control injections with either heat-inactivated microsporidia (heated Ha-Msp) isolated from *Harmonia* (dashed line) or PBS alone (dotted line) showed statistically significant differences ($P < 0.05$), indicating that mortality of *Coccinella* is predominantly caused by living microsporidia. Circles indicate where animals have been taken out of the experiment to control the presence of microsporidia in the hemolymph. Cs, *Coccinella septempunctata*; PBS, phosphate-buffered saline.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/340/6134/862/DC1
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Figs. S1 to S4
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Global catches, exploitation rates, and rebuilding options for sharks

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ABSTRACT

Adequate conservation and management of shark populations is becoming increasingly important on a global scale, especially because many species are exceptionally vulnerable to overfishing. Yet, reported catch statistics for sharks are incomplete, and mortality estimates have not been available for sharks as a group. Here, the global catch and mortality of sharks from reported and unreported landings, discards, and shark finning are being estimated at 1.44 million metric tons for the year 2000, and at only slightly less in 2010 (1.41 million tons). Based on an analysis of average shark weights, this translates into a total annual mortality estimate of about 100 million sharks in 2000, and about 97 million sharks in 2010, with a total range of possible values between 63 and 273 million sharks per year. Further, the exploitation rate for sharks as a group was calculated by dividing two independent mortality estimates by an estimate of total global biomass. As an alternative approach, exploitation rates for individual shark populations were compiled and averaged from stock assessments and other published sources. The resulting three independent estimates of the average exploitation rate ranged between 6.4% and 7.9% of sharks killed per year. This exceeds the average rebound rate for many shark populations, estimated from the life history information on 62 shark species (rebound rates averaged 4.9% per year), and explains the ongoing declines in most populations for which data exist. The consequences of these unsustainable catch and mortality rates for marine ecosystems could be substantial. Global total shark mortality, therefore, needs to be reduced drastically in order to rebuild depleted populations and restore marine ecosystems with functional top predators.

1. Introduction

Sharks, skates, rays and chimaeras together comprise the chondrichthyan fishes (Class *Chondrichthyes*), a group of about 1000 species that has persisted for at least 400 million years, rendering them one of the oldest extant vertebrate groups on the planet. Recently, however, the global growth of fishing, coupled with *Chondrichthyes*' relatively slow growth and reproductive rates, have resulted in the progressive depletion of populations around the world. This trend has been particularly pronounced for sharks, largely due to their inherent vulnerability, and an increasing demand, particularly for their fins, in the Asian market [1–4]. As such, many shark species are comparable to great whales, which also have late maturity, slow growth and low reproductive rates, and experienced escalating global fishing pressure until a global whaling moratorium came into effect in 1986 [5]. Similar to whales, quantifying the precise extent of sharks' decline, the risk of species extinction, and the consequences for marine ecosystems have been challenging and controversial, mostly due to data limitations [4,6–8].

A key problem is the incomplete reporting of shark catches to the United Nations Food and Agriculture Organization (FAO), which tracks the status of fisheries worldwide. Caught sharks are often not landed and are instead discarded at sea [7,9], with such discards not usually reported to national or international management agencies unless there are trained observers on board. Compounding this problem is the practice of shark finning, where the animal's fins are removed prior to the body being discarded at sea [9]. Due to the high value of the fins in Asian markets this practice is globally widespread. Some jurisdictions, such as Canada, the United States, Australia, and Europe have gradually introduced anti-finning legislation over the last 10 years, yet the practice continues in most other parts of the world [2]. Therefore it is very likely that reported catches represent only a fraction of total shark mortality. For example, Clarke et al. [9] used

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trade auction records from Hong Kong to estimate that the total mass of sharks caught for the fin trade. Estimates ranged between 1.21 and 2.29 Mt (million metric tons) yr^{-1} with a median estimate of 1.70 Mt yr^{-1} in the year 2000. This amounted to more than four times the reported shark catch from FAO at that time [9].

Notwithstanding these problems, the FAO, among other management bodies, has long recognized the conservation challenges associated with sharks and their relatives, and it launched an International Plan of Action for Sharks in 1999 (IPOA-Sharks, which also includes skates, rays, and chimaeras). This plan aims to enhance the conservation and management of sharks and their sustainable use, while improving data collection and the monitoring and management of shark fisheries [10]. The IPOA-Sharks further recommends that all states contributing to fishing mortality on sharks should participate in its management, and should have developed a National Shark Plan by 2001. However, progress remains disappointing so far, with limited adoption and implementation of IPOA goals at the national level [2,11].

The objective of this paper is to provide an up-to-date assessment of the current status of shark populations including estimated global catches, current exploitation rates (herein defined as the total catch divided by the estimated biomass), and potential extinction risks at current levels of exploitation. Based on this review, possible management solutions for conserving and rebuilding shark populations are discussed. The authors intend to provide critical baseline information for the further development of national and international action plans that help ensure the conservation of sharks and their relatives.

2. Methods

Available information to estimate total shark fishing mortality, including reported landings, dead discards, and illegal, unregulated and unreported (IUU) landings were compiled for this paper. Caught sharks are either landed (reported or IUU) or discarded (alive or dead). Discarded sharks that are finned suffer 100% mortality, and those that are not finned suffer a lower post-release mortality [12]. These components (reported and IUU landings, dead discards) are estimated here from published data. In some cases it was necessary to convert shark numbers to weights or vice versa. To this end published estimates of average shark weights for species belonging to four major species groups were extracted from the available peer-reviewed literature: pelagic (e.g. *Prionace glauca*, *Isurus oxyrinchus*), large coastal (e.g. *Galeocerdo cuvier, Carcharhinus leucas*), small coastal (e.g. *Squalidae, Squatina spp.*), and deep water sharks (e.g. *Centrophorus granulosus, Apristurus profundorum*). Published weights from each study were averaged by species group in each study (e.g. all pelagic species weights were combined into one estimate), and then the median weight was computed across studies.

Reported catches were derived from the 'Fishstat' FAO online landings database [13]. FAO results were also compared with the 'Sea Around Us Project' (SAUP) database at the University of British Columbia, which is based on the FAO data and additional sources [14]. Since results were similar ($<10\%$ difference in catches), and temporal coverage was more complete (1950–2010) for the FAO data, the latter was used for analysis. Chondrichthyan catches included the following categories: large coastal and pelagic sharks, small coastal sharks, deep-water sharks, undifferentiated sharks, rays and chimaeras (mixed group), rays, skates, chimaeras (separate groups) and undifferentiated skates and rays. To estimate the total take of sharks, the proportion of sharks relative to other chondrichthyan catch from the differentiated groups was determined, and it was assumed that it was the same as in the undifferentiated (mixed species) group. Global trade data for shark fins were extracted and summarized from the same data base. For regional comparison, we also analyzed trade data from the Government of Hong Kong Department of Aquaculture and Fisheries Census and Statistics Reports.

The extent of illegal, unregulated and unreported (IUU) catch was estimated from the peer-reviewed literature [15] by taking the average of the low (11 Mt yr^{-1}) and high estimates (26 Mt yr^{-1}) for global IUU fishing, equivalent to 18.5 Mt yr^{-1}. Since the proportion of chondrichthyans in the IUU catches is unknown, it was assumed that chondrichthyans comprise the same proportion in the IUU catch as they do in the reported catch (1.2% on average). This is likely conservative because shark catches are often unreported, for example in artisanal or bycatch fisheries. When converting IUU catches to numbers of individuals it was also assumed that the proportional representation of major species groups was similar to the reported catch.

The amount of discarded sharks was estimated from published data, where scientifically trained observers had determined the overall catch rates for sharks in commercial fisheries. This analysis was performed comprehensively for the global longline fleet, a major fishery that operates worldwide and is well-known for its high proportion of shark bycatch and discards [3]. First the rate of shark catch was estimated from published sources for each major ocean basin, then this was scaled up by using the reported global longline effort, estimated at 1.4 billion hooks for the year 2000 [16]. Global effort and catch rate data were not available for other fishing gears that catch sharks (e.g. gillnet, purse-seine, troll, and trawl). Hence it was assumed that the proportion of longline shark catch in the total global shark catch would be the same as the proportion of large pelagic sharks in the total reported catch, which averaged at 52%. This assumption is based on the rationale that more than 80% of pelagic sharks caught every year are estimated to be caught on longlines [17]. Furthermore, the proportion of sharks that are finned before being discarded was estimated, along with the proportion of sharks that die post-release from other injuries, by compiling and averaging estimates of shark finning and post-release mortality from peer-reviewed published sources.

Furthermore, an average global exploitation rate for sharks was estimated. The exploitation rate is commonly defined as the total catch divided by the total biomass. Only one published estimate of total biomass was available, which amounts to 86.3 Mt for all elasmobranchs (sharks, rays, skates) combined [18]. It was assumed that half of this biomass (43.2 Mt) is comprised of sharks. The rationale for this assumption is that about half of all elasmobranch species are sharks and about half of the reported elasmobranch landings by weight are sharks. The overall biomass estimate was derived by macro-ecological scaling laws, and as such represents unexploited biomass which does not account for the effects of fishing (methodological details can be found in [18]). Here, it was assumed that half of the original biomass has been depleted due to fishing (21.6 Mt). The rationale for this number is that exploited fish stocks globally are estimated to be at $\sim$30%–45% of their original biomass [19], and 50% is therefore a conservative assumption for a highly exploited group, where many populations have declined 80% or more [20]. The resulting estimate of global shark biomass (21.6 Mt) was used as a basis for estimating global exploitation rate.

Two more independent estimates of exploitation rate were computed here. Published estimates of instantaneous fishing mortality (F) for assessed shark populations were extracted from the global RAM Legacy database of stock assessments [21] and other peer-reviewed sources. These estimates were converted to exploitation rates (U) as follows:

$$U = 1 - \exp(-F), \tag{1}$$

and then averaged across all populations. The second independent estimate of exploitation rate was derived by using the published median estimate of total shark catches for the fin trade, or 1.7 Mt [9], and dividing this by the total biomass estimate

derived above. Note that this procedure is again conservative. It assumes that all shark mortality arises from the fin trade, and no extra mortality occurs.

Finally, observed exploitation rates in individual fisheries were compared here against the intrinsic rebound potential of exploited shark populations. The rebound potential represents the maximum rate of increase (r) of a population given its life history characteristics (average annual fecundity of females, maturity age, maximum age, natural mortality rate), and hence its ability to withstand fishing or recover from excessive fishing mortality under ideal environmental conditions. Estimates of r for individual shark species were obtained from Smith et al. [22] or calculated using the methods outlined in Smith et al. for 62 shark species where adequate life history data existed. The proportion of shark populations where the realized rate of fishing mortality exceeded its rebound potential was calculated from these data. Those species where the exploitation rate exceeded the rebound rate were deemed at risk of further depletion and extinction.

3. Results

Each year, global landings of sharks and other fisheries resource species are reported by fishing states to the FAO (Fig. 1). Since 1950, *Chondrichthyes* (sharks, rays, skates and chimaeras) have comprised between 1% and 2% of the total landings (Fig. 1A, average proportion of 1.2%). Sharks made up about half of the total *Chondrichthyes* landings over that time frame (Fig. 1B). Both shark and total *Chondrichthyes* landings have risen sharply from 1950s to the late 1990s, and have since declined slightly (Fig. 1B). Over this time frame, shark landings have increased 3.4-fold from 120,677 t in 1950 to 414,345 t in 1997, and since then have declined by 7.5% to 383,236 t in 2010. By comparison, the reported landings of skates, rays, and chimaeras increased 3.6-fold over the same period, peaking at 556,470 t in 2003, but since declined by 26.5% to 353,549 t in 2010. As such, *Chondrichthyes* landings showed a trajectory that is similar to global fish landings, which experienced a steady increase from 1950s to 1990s followed by a slow decline (Fig. 1A); however, *Chondrichthyes* displayed a later peak than global landings, and a sharper decline since that peak (Fig. 1B).

Regionally, from the 1990s until the present day, reported landings of sharks and their relatives have remained approximately stable in Europe, the Americas and Oceania, while they have increased in Africa, and fallen in Asia, which on average accounted for 52% of *Chondrichthyes* landings worldwide (Fig. 1C). While reported landings have generally been stable or declining, the trade volume of shark fins appears to have sharply increased since the late 1980s. No apparent evidence was found of a decline

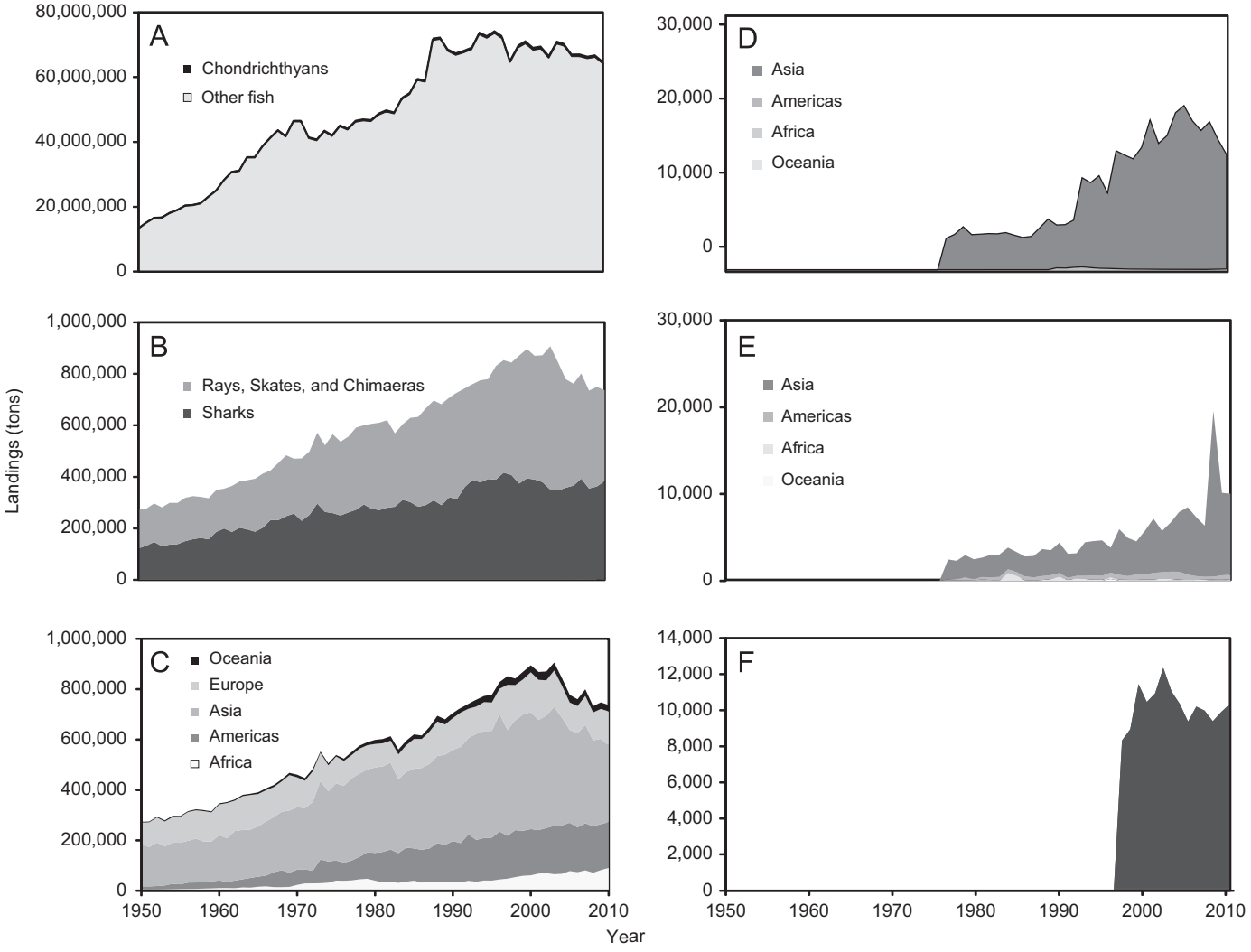

Fig. 1. Global landings trends. (A) Reported landings of wild-caught bony fish and *Chondrichthyes*, as derived from FAO landings data. (B) Reported FAO landings of sharks versus other *Chondrichthyes* (rays, skates and chimaeras). (C) Reported landings of *Chondrichthyes* by region. (D) Trade in shark fin imports and (E) exports as reported by FAO. (F) Trade data for shark fin imports to Hong Kong as reported by the Government of Hong Kong Department of Aquaculture and Fisheries.

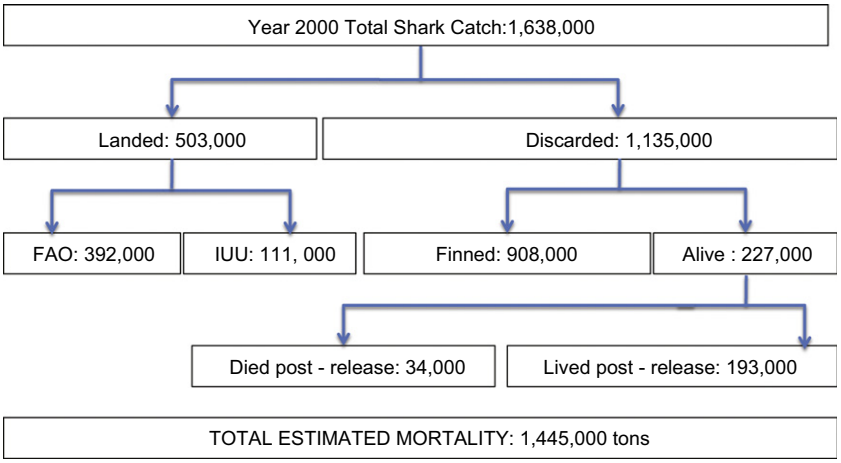

Fig. 2. Estimating global shark mortality for the year 2000. Included are reported (from FAO) and illegal, unreported, and unregulated (IUU) landings as well as shark discards. Total mortality was calculated as the total catch minus the number of sharks which survived discarding. All figures were rounded to nearest 1000 metric tons.

in shark fin imports (Fig. 1D) or exports (Fig. 1E) following the establishment of finning bans in the mid-1990s. This observation appears corroborated by the lack of a downward trend in trade data for shark fins imported into the major Hong Kong market (Fig. 1F). Thus finning regulations do not appear to have reduced the volume of fins traded in global or regional markets. According to FAO commodity figures, the total import value of shark fin products ranged from about USD 20 million in 1976 to a high of USD 455 million in 2000, and has since fluctuated between USD 306 and 419 million.

Our estimates of total shark catches for the year 2000 including reported and unreported landings and discards are provided in Fig. 2. Reported landings from the FAO database totaled 392,226 t in that year. Global illegal, unregulated and unreported (IUU) catches (excluding discards and artisanal catches) were estimated to average 18.5 Mt for the year 2000 [15]. It was assumed that similar to the reported catches *Chondrichthyes* also made up 1.2% of IUU landings (222,000 t), and sharks made up half of that, or 111,000 t. Hence, total shark landings (reported plus estimated unreported) in 2000 were estimated at about 503,000 t (Fig. 2).

To account for discards, the average catch per unit of effort (CPUE) for sharks caught on pelagic longlines was estimated from a number of published sources (Table 1), which yielded average catch rates of 16.5 (Pacific), 21.2 (Atlantic) and 4.3 (Indian Ocean) sharks caught per 1000 hooks. The global effort of longline fishing in the year 2000 was estimated at 1.4 billion hooks [16] with 728 million hooks set in the Pacific, 518 million in the Atlantic, and 154 million in the Indian Ocean. Multiplied by the ocean-specific catch rates (Table 1), these figures represent a longline shark catch of about 23,656,000 individuals, or 852,000 t assuming 36 kg average weight for pelagic sharks (Table 2). Pelagic sharks made up 52% of the identified shark catch in the FAO data, as opposed to coastal and deepwater sharks (48% of identified catch). Hence it was assumed that the estimate derived above from pelagic longlines (852,000 t) represents about 52% of the total catch. This raised the total catch estimate for all fishing gears to 1,638,000 t (Fig. 2). When the estimated landed catch (503,000 t) was subtracted, a global estimate of total shark discards (1,135,000 t) was derived.

According to available data (Table 3) the average rate of shark finning in 2000 was 80%. This high percentage was likely due to the high demand for the fins, their high value, as well as the lack of effective finning regulations in most fishing areas. Thus it was estimated that 80%, or 908,000t of discarded sharks, were finned, while the remainder (227,000 t) were released alive. A proportion of the sharks that are released alive suffer post-release mortality due to injury and stress. Published estimates of post-release mortality are in the order of 15% or higher [12,23]. Thus it was assumed here, that 15% of released (non-finned) sharks died from fishing-related injuries (34,000 t) and 85% survived (193,000 t). Combining reported and unreported catches, as well as dead or moribund discards, the total fishing mortality for sharks in 2000 was estimated here at 1,445,000 t (Fig. 2). Out of this, 1,409,000 t of landed catch plus finned discards were available to supply the fin trade. This is close to the independently derived median estimate for the 2000 shark fin trade of 1,700,000 t [9].

Using the average shark weights given in Table 2, these masses were converted into numbers of sharks. Using the median estimate of 20.8 kg for all sharks (Table 2), it was here calculated that the total mortality of 1,445,000 t translates into 69,471,000 shark individuals. However, accounting for the fact that the species composition of the FAO catch is partly known (in 2000: 82,582 t small coastal species, 111,858 t large pelagic, 5004 t deepwater species, and 182,782 t unidentified) and that these groups have known average weights (see Table 2, and assuming 20.8 kg for unidentified species), it was calculated that at least 49,011,000 sharks comprised the FAO reported landings in 2000. Assuming 20.8 kg per shark for the remaining catch (IUU and discarded dead sharks) a conservative mortality estimate of 99,618,000 sharks in 2000 was computed. This value is sensitive to our estimated average weights and species composition of the shark catch derived from published data. For example, one might assume that the species composition of the FAO species-identified catch also applies to the unidentified sharks reported to FAO; this would yield 74,321,000 sharks in the FAO catch, and 124,928,000 sharks in total including IUU and discards. Or one might assume the same species composition for the IUU catch; under this scenario the total mortality estimate increases to 140 million individuals. When assuming that both IUU and discards have a catch species composition similar to the reported FAO catch, this total estimate increases to 273 million sharks.

It is unclear how these figures might have changed since 2000, given changes in finning legislation in several jurisdictions (e.g. USA, Canada, Europe, and Australia) and the recent establishment of shark sanctuaries in others (Palau, Maldives, Honduras, Bahamas, and Tokelau). From 2000 to 2010 the FAO landings of sharks declined only slightly (by 2.3%) to 383,236 t. Assuming that both discards and IUU fishing declined by a similar fraction between 2000 and 2010, one would estimate total mortality in 2010 at 1,412,000 t, or between 97 and 267 million sharks, depending on the chosen scenario of species composition and average weights.

Using the above estimates, combined with independent figures, a total exploitation rate U (catches over biomass, in percent per year) for global shark populations was calculated (Table 4).

Table 1
Observed catch per unit effort of sharks in longline fisheries.

Fishery	Ocean	Region	Year	CPUE	Hooks	Ref.
Swordfish	Pacific	Southeast	2001–2006	6.9	155,060	[37]
Swordfish	Pacific	Eastern Central	1994–2006	16.7	NA	[38]
Swordfish and tuna	Pacific	Southeast	2004	3.6	72,090	[39]
Swordfish and sharks	Pacific	Northwest	2005	38.7	19,800	[40]
Swordfish and sharks	Pacific	Northwest	2005	91.1	28,800	[40]
Swordfish and sharks	Pacific	Northwest	2002–2003	47.8	36,480	[41]
Tuna	Pacific	Eastern Central	2006	2.6	180,000	[42]
Tuna	Pacific	Western Central	2005–2006	2.3	75,101	[43]
Tuna	Pacific	Southwest	1990–1998	7.5	12,725,046	[44]
Tuna	Pacific	Western Central	2005–2009	3.6	NA	[45]
Tuna	Pacific	Western Central	2005–2008	1.2	95,150	[46]
Tuna	Pacific	Eastern Central	1994–2006	2.2	NA	[38]
Tuna	Pacific	Eastern Central	2005–2006	3.4	2,773,427	[47]
Tuna and billfish	Pacific	Western Central	2005	3.3	44,100	[48]
Sharks	Pacific	Eastern Central	2004	25.2	15,200	[49]
Sharks	Pacific	Eastern Central	2005–2006	60.0	18,800	[50]
Mahimahi, tuna, billfish and sharks	Pacific	Eastern Central	2007	10.6	43,424	[51]
Mahimahi, tuna and sailfish	Pacific	Eastern Central	1999–2008	4.6	1,974,700	[52]
Mahimahi	Pacific	Eastern Central	2004–2006	10.6	33,876	[53]
Bigeye tuna	Pacific	Western Central	2005–2006	4.4	62,464	[54]
Tuna and billfish	Pacific	Central	1990–1999	7.8	10,944,000	[55]
Average Pacific				16.5		
Swordfish	Atlantic	Southwest	2003–2004	7.2	16,624	[56]
Swordfish	Atlantic	Northwest	2002	31.3	427,312	[57]
Swordfish	Atlantic	Southeast	2000–2005	23.3	447,000	[58]
Swordfish	Atlantic	Western Central	1992–2000	11.1	413,873	[59]
Swordfish	Atlantic	Southeast	1998–2005	2.9	880,000	[60]
Swordfish and tuna	Atlantic	Northwest	2001–2006	18.3	624,854	[61]
Swordfish and tuna	Atlantic	Western Central	2003–2004	5.7	30,600	[62]
Swordfish and tuna	Atlantic	Western Central	1992–2003	10.8	NA	[63]
Swordfish and tuna	Atlantic	Mediterranean	1998–1999	0.5	1,582,000	[64]
Swordfish and sharks	Atlantic	Northeast	2000–2003	32.5	267,109	[65]
Swordfish and sharks	Atlantic	Northeast	2000	14.4	139,500	[66]
Swordfish, tuna and sharks	Atlantic	Southwest	2004–2008	26.7	145,828	[67]
Swordfish, tuna and sharks	Atlantic	Southeast	2000–2005	85.3	8,829,000	[58]
Tuna	Atlantic	Southwest	2006–2007	17.2	7800	[68]
Tuna	Atlantic	Southeast	2000–2005	12.4	71,800	[58]
Tuna	Atlantic	Southeast	1998–2005	15.3	3,520,000	[60]
Tuna	Atlantic	Atlantic	1995–2003	3.4	4,318,119	[69]
Tuna	Atlantic	Eastern Central	2007–2008	2.8	226,848	[70]
Tuna and billfish	Atlantic	Northwest	1990–1999	30.6	1,116,000	[55]
Tuna and billfish	Atlantic	Southwest	2006–2007	2.5	50,170	[71]
Sharks	Atlantic	Northwest	1991–1992	23.6	17,526	[72]
Black scabbardfish	Atlantic	Eastern Central	2009	88.1	4700	[73]
Average Atlantic				21.2		
Swordfish and tuna	Indian	Eastern	NA	3.9	6226	[74]
Swordfish and tuna	Indian	Western	2004–2006	3.6	29,449	[75]
Swordfish and tuna	Indian	Western	2009–2010	11.8	14,112	[76]
Swordfish, tuna and sharks	Indian	Eastern	2004	4.9	3871	[77]
Tuna	Indian	Indian	2004–2008	0.6	14,121,000	[78]
Tuna	Indian	Eastern	2003–2011	2.3	522,992	[79]
Tuna	Indian	Eastern	2005–2011	5.9	38,333	[80]
Tuna	Indian	Eastern	2011	1.2	8375	[81]
Tuna	Indian	East-West	2000–2006	4.9	2,476,148	[82]
Average Indian				4.3		

The global biomass of elasmobranchs before the era of modern fishing was estimated by Jennings et al. [18] as 86,260,000 t. Assuming that half of these elasmobranchs are sharks, a biomass before fishing of 43,130,000 t of sharks was estimated. Conservatively assuming 50% depletion of sharks over the history of modern fishing, a contemporary biomass estimate of 21,565,000 t of sharks was derived. Total mortality was estimated to be 1,445,000 t in 2000 (Fig. 2), which when divided by total biomass, yields an estimated exploitation rate of 6.7% per year (Table 4). Using an alternative mortality estimate of 1,700,000 t, a figure that was independently derived from the fin trade [9], an annual exploitation rate of 7.9% was computed. Averaging across actual exploitation rates from published stock assessments and other sources given in Table 5, an independent estimate of 6.4% exploitation rate was derived. These three estimates are remarkably similar, considering that they were derived by entirely independent sources using different assumptions.

Comparing actual exploitation rates (Table 5; Fig. 3A) to calculated rebound rates of shark populations in general (Fig. 3B), and individual shark populations for which exploitation rates were estimated in particular (Fig. 3C), it was found that exploitation rates (Fig. 3A, Median $U=0.064$) on average exceed the median rebound rates (Fig. 3B, Median $r=0.049$) by about 30%, which is unsustainable over the long term. Notably, the rebound rates for most species were significantly below the three independent estimates of exploitation rates derived in this paper (Table 4). This suggests that the majority of shark populations will continue to decline under current fishing pressure (Fig. 3C).

Table 2
Average shark weights.

Species group	Species	Region	Year	Weight (kg)	Ref.
Large Coastal	2	NE Atlantic	1992–1999	34.0	[83]
Large Coastal	10	North and South Atlantic	2008	58.6	[84]
Large Coastal	2	SW Atlantic	2007–2008	85.0	[70]
Large Castal	5	SW Indian Ocean	1984–2006	46.2	[82]
Large Coastal	11	NW Atlantic	2004	26.5	[85]
Median				46.2	
Pelagic	3	Mediterranean	1998–2001	23.0	[64]·
Pelagic	2	North Pacific	1970–1992	17.0	[86]·
Pelagic	1	South Pacific	1988–1990	8.0	[87]·
Pelagic	6	NW Atlantic	1986–2000	34.0	[88]·
Pelagic	3	North and South Atlantic	1994–2003	38.0	[89]·
Pelagic	3	NW Atlantic	1961–1989	78.0	[90]·
Pelagic	9	North and South Atlantic	2008	76.0	[84]·
Pelagic	4	SW Atlantic	2007–2008	42.0	[70]·
Median				36.0	
Small Coastal	1	SW Atlantic	2005	1.0	[91]
Small Coastal	6	North Aegean Sea	2005–2008	8.0	[92]
Small Coastal	4	NE Atlantic	1992–1999	2.3	[83]
Small Coastal	3	SW Indian Ocean	1984–2006	15.0	[82]
Small Coastal	3	NW/SW Atlantic	1993–2005	2.0	[93]
Median				2.3	
Deep-water	2	NE Atlantic	1993–2000	2.6	[94]
Deep-water	4	North Aegean Sea	2005–2008	11.4	[92]
Deep-water	4	NE Atlantic	1999	5.6	[95]
Deep-water	2	SW Atlantic	2007–2008	3.0	[70]
Deep-water	14	NE Atlantic	1984–1997	9.0	[96]
Median				5.6	
Overall Median				20.8	

Table 3
Published estimates for the proportion of sharks that are finned in various fisheries around the world.

Fishery	Flag	% Finned	Comments	Ref.
Swordfish	USA (Hawaii)	65.0	Pre-regulations (2002)	[38]
Swordfish	Italy	0.0	No market	[38]
Tuna and Swordfish	South Africa	100.0	Pre-regulations (1998)	[38]
Tuna	USA (Hawaii)	76.0	Pre-regulations (2002)	[38]
Tuna	Fiji	84.0		[38]
Tuna	New Zealand	83.8		[44]
Tuna	China, Micronesia	96.8		[45]
Tuna	Unknown	67.8		[97]
Median		79.9		

4. Discussion

The primary goal of this paper was to estimate total catch and fishing-related mortality for sharks worldwide, and to derive an average exploitation rate from these estimates (Table 4). Due to the limited availability of data, particularly for shark discards, this work required a number of assumptions, as detailed above. Yet it allows placement of lower and upper limits on global shark mortality, here estimated to range from 63 to 273 million sharks, with a conservative estimate of ~100 million sharks in the year 2000, or ~97 million in 2010.

At the lower end, one might unrealistically assume that landings reported to the FAO represent all shark mortalities. When accounting for the average weight of different species groups, a minimum estimate of 49 million sharks can be derived from the FAO landings data. Yet this does not account for unreported and illegal catches. If we estimate an average rate of illegal, unregulated and unreported (IUU) fishing, we arrive at a total of 63 million sharks per year for the year 2000. This minimum estimate of global shark mortality changes only slightly from 2000 to 2010 (61 million sharks) as reported shark landings remained near-constant over the decade. This number is also similar to the upper estimate of shark mortality from the fin trade of 73 million individuals [9].

The abovementioned minimum estimate of shark mortality does not include discards and artisanal fishing since these sources of mortality are not accounted for in the FAO and IUU data. In the present paper these numbers are estimated for the first time. While the total catch rate of sharks in global longline fisheries could be well estimated from published data, data of similar quality for other fishing gear types that catch sharks, such as purse seines, gillnets, and trawls, were not available. Hence it was estimated here (from the FAO data) that about 52% of sharks are caught by longlines, with the remaining 48% caught by all other types of gear combined. This likely underestimates the catches of sharks in other fishing gear; trawls for example can catch very large numbers of small coastal sharks, most of which are discarded [7]. Hence the estimate for total mortality including discards is still likely conservative at 100 million sharks in 2000.

These calculations carry uncertainties and should be interpreted with some caution. The number of dead sharks, for example, is sensitive to the assumed percentage of small coastal sharks in the catch. If it is assumed that these are represented in the total catch (including discards) with the same proportion as in the reported and species-identified catch, the total mortality estimate increases to 273 million sharks, which represents an upper limit of shark mortality estimated here. Another uncertain value is the shark mortality from artisanal and recreational fishing, which is only partially accounted for in this analysis, a fact that again renders the estimate of 100-million sharks killed annually conservative.

Finally, the proportion of sharks that are killed for their fins is well known for the early 2000s (Table 3). However a number of regions now have anti-finning legislation that may reduce the incidence of finning and discarding of carcasses, and hence possibly reduce the mortality of sharks. Yet, despite these legislative changes there is presently no apparent sign of leveling off in the global fin trade (Fig. 1D–F). Nor is there much of a decline in the reported global catches of sharks (Fig. 1B).

Several explanations may account for these observations of near-stable catches and fin trade volume. First, fishing effort likely has been geographically displaced over the last decade as the primary fishing grounds supplying the fin trade in the 1990s and early 2000s became increasingly depleted or regulated. Additionally, catch levels may have experienced a certain amount of resiliency if fishers started using other, lower-value species or smaller individuals that were previously discarded. The species composition of the fin trade has not been assessed for more than a decade [9], hence this should become a research priority. Further, the apparent failure of anti-finning laws to curb global mortality may indicate that these laws have yet to be adequately enforced [24]. On the other hand, anti-finning laws primarily address animal welfare and food security issues (i.e. to reduce waste). Although an important first step, these policies are not explicitly designed to reduce catch or ensure sustainability. The premise that anti-finning legislation would contribute to sustainable fisheries rests on the assumption that most fishermen target sharks for their fins only, and would refrain from targeting sharks if they had to retain the carcass. This assumption is weak. Many

Table 4
Summary statistics for the exploitation of global shark populations.

Measure	Year	Estimate	Unit	Comments	Ref.
Removals					
All Elasmobranchs	2000	894,802	tons	Reported catch only	[13]
Sharks	2000	392,226	tons	Reported catch only	This study
Sharks	2000	38,000,000	Individuals	Fin trade only (median estimate)	[9]
Sharks	2000	1,700,000	tons	Fin trade only (median estimate)	[9]
Sharks	2000	99,617,577	Individuals	All sharks, total mortality	This study
Sharks	2000	1,444,847	tons	All sharks, total mortality	This study
Biomass and abundance					
Elasmobranch biomass	NA	86,260,000	tons	Sharks and rays, before fishing	[18]
Shark biomass	NA	43,130,000	tons	Assuming 50% sharks	This study
Shark biomass	2000	21,565,000	tons	Assuming 50% depletion by 2000	This study
Shark abundance	2000	1,036,778,846	Individuals	Assuming 20.8 kg/shark	This study
Exploitation rate					
Shark exploitation rate	2000	6.7	Percent/yr	Based on total biomass	This study
Shark exploitation rate	2000	7.9	Percent/yr	Based on fin trade statistics	[9]
Shark exploitation rate	2000	6.4	Percent/yr	Based on assessments	This study

Table 5
Published values of instantaneous fishing mortality (F) and exploitation rate (U) for assessed shark populations.

Species name	Common Name	F	U	Ref.
Rhizoprionodon terraenovae	Atlantic sharpnose	0.460	0.369	[98]
Squalus acanthias	Spiny dogfish	0.206	0.186	[21]
Carcharhinus porosus	Smalltail	0.193	0.176	[99]
Sphyrna tiburo	Bonnethead	0.187	0.171	[21]
Sphyrna lewini	Scalloped Hammerhead	0.160	0.148	[100]
Prionace glauca	Blue	0.160	0.148	[101]
Alopias pelagicus	Pelagic thresher	0.150	0.139	[102]
Carcharhinus plumbeus	Sandbar	0.130	0.122	[103]
Carcharhinus plumbeus	Sandbar	0.123	0.116	[21]
Lamna nasus	Porbeagle	0.090	0.086	[104]
Isurus oxyrinchus	Shortfin mako	0.066	0.064	[105]
Triakis semifasciata	Leopard	0.061	0.059	[106]
Lamna nasus	Porbeagle	0.056	0.054	[104]
Carcharhinus obscurus	Dusky	0.053	0.052	[107]
Prionace glauca	Blue	0.047	0.046	[101]
Carcharhinus limbatus	Blacktip	0.041	0.04	[21]
Carcharhinus acronotus	Blacknose	0.031	0.031	[21]
Isurus oxyrinchus	Shortfin mako	0.028	0.028	[108]
Prionace glauca	Blue	0.020	0.02	[108]
Carcharhinus limbatus	Blacktip	0.003	0.002	[21]
Carcharhinus isodon	Finetooth	0.001	0.001	[21]
Median		0.066	0.064	

countries consume shark meat [25] and fishermen opt to land whole sharks, even if the meat is not as valuable as the fins. Several at-risk shark species are generally kept rather than being finned in certain pelagic fisheries where freezer space is limited [24].

It is not surprising that anti-finning measures have been introduced widely given the intense public pressure that arose, especially since anti-finning laws are more palatable to industry than stringent catch reductions when local markets for the meat exist. In contrast, the monitoring, assessment and enforcement capacity required to sustainably manage shark fisheries is often perceived by regulatory agencies as being prohibitively costly relative to the simple adoption of anti-finning legislation. Regardless, some nations have recently invested in sustainable shark fisheries management, introducing catch limits, effort control, time-area closures, and other protective measures for the most vulnerable species. In some cases, such local measures appear to have been successful in halting declines [8]. The findings reported here highlight the fact that shark conservation policies generally need to focus on sustainability, as there is no evidence that a

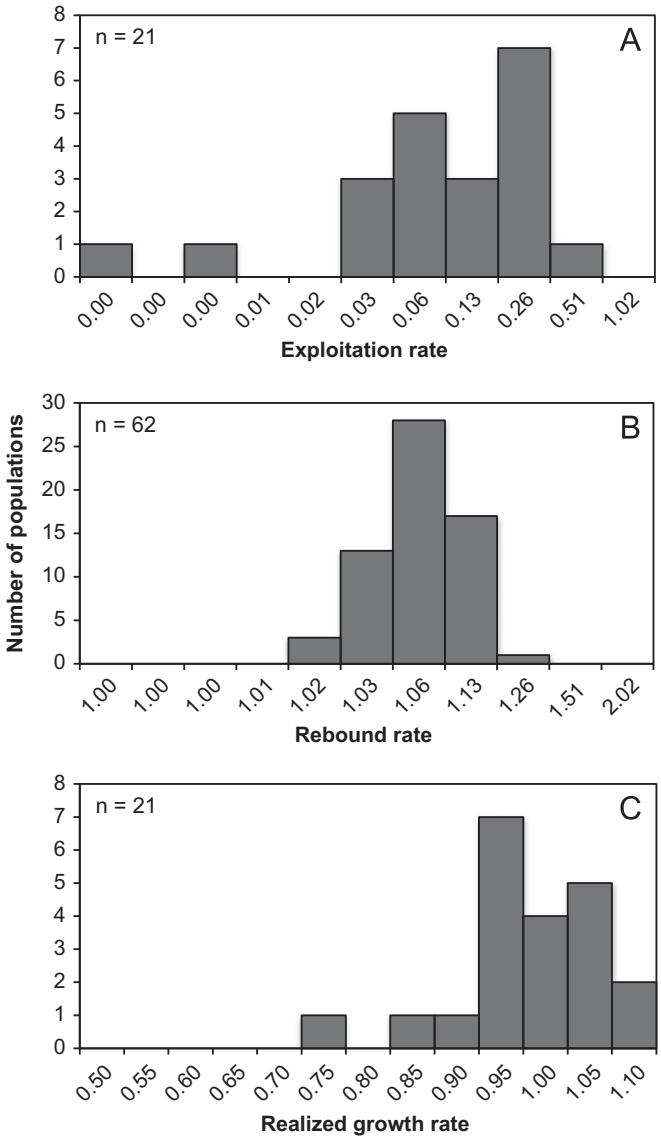

Fig. 3. Exploitation rates versus rebound potential of shark populations. (A) Exploitation rates of 21 assessed populations (in % of biomass exploited per year). (B) Maximum potential rebound rate (% increase per year) of 62 species with available data. (C) Realized growth rates calculated by subtracting exploitation rate from the maximum rebound potential (declining populations are <1.00).

Table 6
IUCN extinction risk status of global shark populations (CR=critically endangered, EN=endangered, EN=endangered, VU=vulnerable, LC=least concern, DD=data deficient).

Order	CR	EN	VU	NT	LC	DD	ALL
Carcharhiniformes	7	10	21	38	67	120	263
Heterodontiformes	0	0	0	0	4	5	9
Hexanchiformes	0	0	0	3	0	2	5
Lamniformes	0	0	10	1	2	2	15
Pristioforiformes	0	0	0	1	3	2	6
Orectolobiformes	0	0	7	11	8	12	38
Squaiformes	1	0	6	13	35	63	118
Squatiniformes	3	4	4	1	2	5	19
Total species	11	14	48	68	121	211	473
Percentage of assessed	2.3	3.0	10.1	14.4	25.6	44.6	100
Percent of non-DD	4.2	5.3	18.3	26.0	46.2	NA	NA

legislative focus on anti-finning has reduced global landings and shark mortality rates.

From a legislative perspective, an important question to consider is what proportion of shark species may be at risk from extinction? According to the International Union for the Conservation of Nature (IUCN) Shark Specialist Group, 28% of assessed and non-data deficient shark species are globally at risk of extinction, i.e. classed as vulnerable, endangered or critically endangered (Table 6). A small number of these species are now receiving protection through national and international agreements. The white shark, whale shark, and basking shark, for example, are protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). From the analyses presented here, a larger proportion of species appear to be at risk. According to available assessments, 48% of exploited shark populations were fished above their rebound rate, and 68% of species had rebound rates that were below the median global exploitation rate (6.7%). While these are rough generalizations based on global averages, it is here noted that the IUCN Specialist group results (Table 6) seem conservative, when compared to an analysis of exploitation rates (Fig. 3). Note that the actual status of individual species varies by region, and is influenced by local regulations, targeting practices, and effort allocation (e.g. [8]).

Beyond these species-level risks, there are concerns about the potential ecosystem consequences of depleting shark populations. Fortunately, there are a growing number of empirical studies that address the ecological consequences of declines in shark populations, which vary across taxa and ecosystems [1,6]. Time series data suggest that wider community rearrangements often follow declines in shark populations [1] and that the removal of large-bodied coastal sharks that prey upon other large-bodied taxa are likely to have cascading consequences for highly productive coastal ecosystems that support other fisheries [6,26]. Lower impacts of shark removals have been predicted by models for some small coastal species [27] and pelagic sharks, which may fill similar niches to billfish and tuna [28]. More broadly, however, across multiple environments on land, in lakes, rivers, and in the sea, the removal of large-bodied predators is commonly associated with large-scale changes in ecosystems [29]. Therefore, a precautionary approach should apply to shark management. The loss, especially of larger apex predators, could and has led to unexpected disruptions of ecosystems and non-shark fisheries [30].

Given the results of this paper, and much previous work on the vulnerability of sharks to overfishing, it is imperative that robust strategies for shark management and conservation be designed. This was formally recognized by the FAO in 1999, when it published an International Plan of Action for Sharks (IPOA-Sharks), a voluntary policy instrument within the framework of the Code of Conduct for Responsible Fisheries [10]. Although all concerned states are encouraged to implement it, progress at the national level has been slow [11], and concerns over the possible extinction of vulnerable species are mounting [2,3,31]. In a recent paper [29], evidence for the rebuilding of depleted elasmobranch populations under management was evaluated and these authors found little general support as of yet that rebuilding was occurring [32].

At the same time it appears that the demand for shark fins remains high (Fig. 1D–F), and there is a general concern that localized protective measures just displace the problem into less regulated areas, including many developing countries and the high seas [19]. Existing finning bans are an important first step, but they may be ineffective at reducing overall shark mortality, as there is no evidence that global shark catch or shark fin trade is declining. Given the failure to effectively reduce the unsustainable mortality of sharks on a global scale, there appears a need for a more binding international agreement on the protection of sharks. This could be similar to what has been done for the global conservation of whales through the establishment of the International Whaling Commission [5]. In that case, a globally threatened group of large marine animals was effectively saved from extinction by imposing stringent global catch regulations, and ultimately a global moratorium on commercial whaling.

If the goal was to at least partially rebuild depleted shark populations worldwide, what actions would be required? Caddy and Agnew [33] and Worm et al. [34] have discussed management options that exist for rebuilding fish populations, and analyzed the empirical evidence for successful recovery; Ward-Paige et al. [32] recently reviewed the same issue for sharks. These authors concluded that rebuilding depleted stocks is demonstrably possible, and occurs where a number of management instruments are combined to reduce mortality to an appropriately low level [32–34]. This level depends both on the status of the stock, and its productivity, or rebound potential [33]. As most shark populations have low productivity compared to other fish stocks, and stock status is typically poor or unknown, the case for ensuring a large decrease in catches and the establishment of a moratorium on fishing appears strong [32,33]. In the absence of a complete moratorium, the rebuilding of depleted shark populations requires very stringent controls on exploitation rates, the enforcement of appropriately low mortality rates, the protection of critical habitats, monitoring, and education [32]. Such controls have been implemented with some success in parts of the United States, for example [8], but would be more difficult to enforce elsewhere [15,19,35]. Given that the costs of these measures can be considerable and are currently carried by tax payers in shark fishing nations, some of this burden could be shifted to the shark fishing and fin export industries. Shark fins are a luxury product [25], which means that demand is unlikely to be curbed by modest price increases. Thus, imposing taxes on the export or import of shark fins will generate income that could be directed to these domestic shark fisheries management efforts.

Another option is to focus on the most vulnerable species, particularly those that are heavily affected by the global fin trade. CITES currently protects three of the most charismatic species, the whale, basking, and white sharks. These species are well-known and support large dive and ecotourism industries [36] hence there is also an economic incentive for their protection. Many other species, however, are of similar conservation concern [3], yet their attempted listing under CITES has so far failed due to opposition from shark-fishing and -consuming countries. In any case, trade bans for the most depleted species need to be combined with scientifically-based catch limits, and appropriately-sized

protected areas, such as the shark sanctuaries recently established by a handful of developing nations. Given the continuing high trade volume for shark fins (Fig. 1D–F), large unreported catches and discards (Fig. 2), and excessive exploitation rates (Fig. 3), it is here suggested that protective measures have to be scaled up significantly in order to avoid further depletion and the possible extinction of sharks, with likely severe effects on marine ecosystems around the world.

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