Nikon Optiphot. After placement on a cover slip, it was overlain with agar, and subsequently osmicated, dehydrated and embedded as described. Sections of 1 μm in thickness were examined with a High Voltage Electron Microscope (HVEM) at 1 MeV. SEM of flagellates and ciliates involved fixation of ~1 ml sediment in 1% OsO_4 vapour for 1 hour, subsequent concentration onto 3-μm filters, and dehydration with ethanol. Filters were then critical-point dried, coated with Au and examined with a LEO 982.

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Mortality of sea lions along the central California coast linked to a toxic diatom bloom

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Over 400 California sea lions (*Zalophus californianus*) died and many others displayed signs of neurological dysfunction along the central California coast during May and June 1998. A bloom of *Pseudo-nitzschia australis* (diatom) was observed in the Monterey Bay region during the same period. This bloom was associated with production of domoic acid (DA), a neurotoxin[1] that was also detected in planktivorous fish, including the northern anchovy (*Engraulis mordax*), and in sea lion body fluids. These and other concurrent observations demonstrate the trophic transfer of DA resulting in marine mammal mortality. In contrast to fish, blue mussels (*Mytilus edulus*) collected during the DA outbreak contained no DA or only trace amounts. Such findings reveal that monitoring of mussel toxicity alone does not necessarily provide adequate warning of DA entering the food web at levels sufficient to harm marine wildlife and perhaps humans.

Harmful algal blooms (HABs) pose serious public health threats and their economic impact can total hundreds of millions of dollars annually[2,3]. HABs are also associated with mortality of wildlife, including birds and marine mammals[4–7]. Establishing an unambiguous connection between HABs and marine mammal mortality is difficult. Lack of background data on the sensitivity of these animals to algal toxins, as well as challenges associated with ascribing a definitive cause of death, can make proof of HAB-related marine

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mammal mortality elusive[8]. Logistical problems associated with detecting harmful algae and their toxins over large spatial and temporal scales also hamper efforts to map blooms and provide a context within which to evaluate concurrent mortality events. Here, *Pseudo-nitzschia* species-specific DNA probes[9–11] and a DA receptor binding assay[12] were used to detect a toxic algal bloom. We also employed liquid chromatography-tandem mass spectroscopy (LC-MS/MS) to confirm the presence of DA in plankton, anchovy, and in sea lions that died during the bloom, and histopathology to show brain lesions characteristic of DA poisoning.

The diatoms observed in Monterey Bay in the early spring of 1998 consisted primarily of *Chaetoceros* spp. During this period, species of *Pseudo-nitzschia* were largely absent and plankton samples did not contain detectable DA activity (see Fig. 1a–c, 11 March–20 April 1998). During the first half of May there was a sudden and sharp rise in the abundance of *P. australis* and in DA activity (Fig. 1a, b). The maximum recorded density of *P. australis* was $\sim 1.3 \times 10^5$ cells l^{-1}. At the height of the bloom, plankton were concentrated in a narrow band along the shore (Fig. 2a) and may have been entrained within, or responded to, a water mass enriched with silicate, a chemical signature indicative of terrestrial freshwater runoff[13]. This pattern is evident in nutrient levels (Fig. 1d) observed at the Santa Cruz sampling station during the initiation phase of the bloom[14]. Potential links between *Pseudo-nitzschia* blooms and

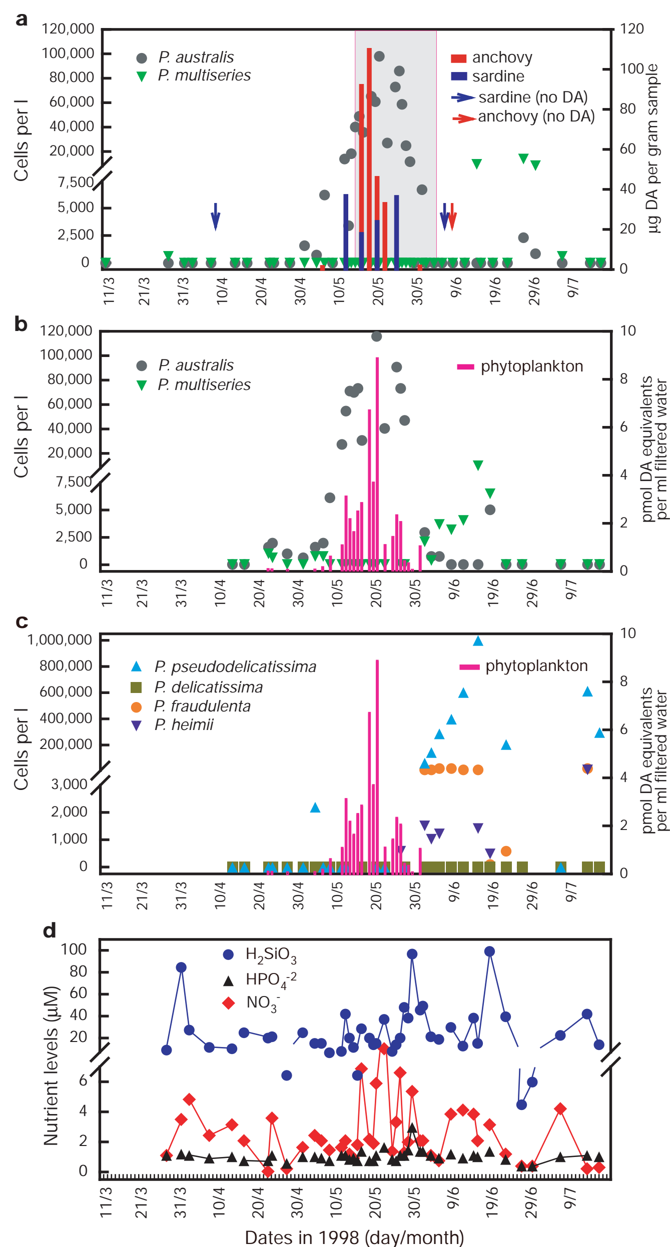

Figure 1 Abundance of *Pseudo-nitzschia* species, DA activity in plankton and fish, and nutrient concentrations. Data taken at Sant Cruz wharf from 11 March–16 July 1998. **a**, *P. australis* and *P. multiseries* detected by sandwich hybridization[10,11]. Bars, μg DA per gram sardine and anchovy determined by HPLC–UV (ref. 1; S.L. and G. Langlois, personal communication); arrows, sardine and anchovy collected, DA not detected; shaded area period of sea lion mortality in Monterey Bay. **b**, *Pseudo-nitzschia* species detected by whole-cell hybridization[9]. Bars, receptor assay-based DA activity[12] in plankton. **c**, *Pseudo-nitzschia* species detected by whole-cell hybridization[9]; DA as in **b**. **d**, Concentrations of silicate (H_2SiO_3), nitrate (NO_3^-) and phosphate (HPO_4^{-2}) (ref. 23).

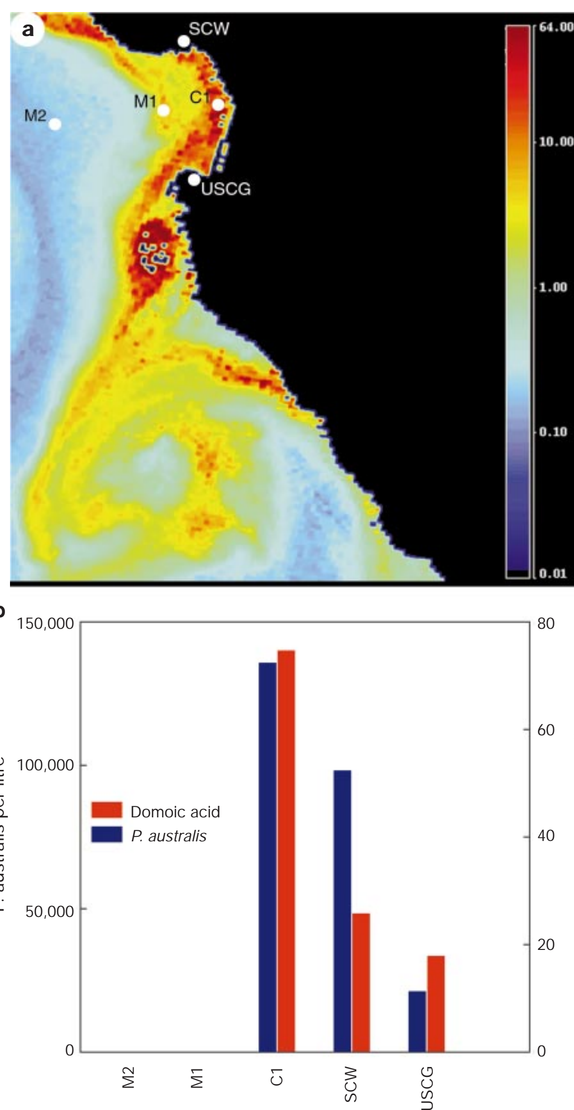

Figure 2 Satellite image of Monterey Bay, California, *P. australis* cell density and DA activity. **a**, 15 May 1998, estimate of chlorophyll *a* inferred from SeaWiFS image of the central California coast produced using SeaDAS with NASA standard algorithms[26]. Scale is μg l^{-1} provided for qualitative purposes only as calibration is on-going (http://www.mbari.org/oasis/index.html). Shown are the Santa Cruz wharf (SCW) and the United States Coast Guard pier (USCG), C1, M1 and M2, ship and mooring-based sampling sites. **b**, Distribution of *P. australis*[10,11] and per cell estimates of DA activity[12] (Table 1) on 20 May 1998, at locations shown in **a**.

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Table 1 Results of the domoic acid (DA) receptor binding assay for phytoplankton, anchovy and sea lion matrices

Sample identification*	Sample matrix	DA equivalents†	LC-MS/MS‡
SCW 9 May 1998	Phytoplankton	31.18 pg cell^{-1}	
SCW 12 May 1998	Phytoplankton	16.10 pg cell^{-1}	
SCW 14 May 1998	Phytoplankton	7.22 pg cell^{-1}	
SCW 18 May 1998	Phytoplankton	20.83 pg cell^{-1}	positive
C1 20 May 1998	Phytoplankton	74.59 pg cell^{-1}	
SCW 25 May 1998	Phytoplankton	8.43 pg cell^{-1}	
Anchovy 22 May 1998a	Anchovy gut	71.30 μg g^{-1}	positive
Anchovy 22 May 1998b	Anchovy gut	69.67 μg g^{-1}	
Anchovy 4 June 1998a	Anchovy gut	2.53 μg g^{-1}	
Anchovy 4 June 1998b	Anchovy gut	0.27 μg g^{-1}	
CSL 3734 22 May 1998	Sea lion serum	0.20 μg ml^{-1}	nd
CSL 3724 21 May 1998	Sea lion serum	0.17 μg ml^{-1}	
CSL 3707 20 May 1998	Sea lion urine	3.72 μg ml^{-1}	
CSL 3794 25 May 1998	Sea lion urine	1.40 μg ml^{-1}	
CSL 3749 22 May 1998	Sea lion urine	0.72 μg ml^{-1}	
CSL 3741 23 May 1998	Sea lion urine	0.03 μg ml^{-1}	
CSL 3800 26 May 1998	Sea lion urine	0.49 μg ml^{-1}	
CSL 3726 21 May 1998	Sea lion urine	0.12 μg ml^{-1}	
CSL 3783 23 May 1998	Sea lion faeces	182.01 μg ml^{-1}	positive
CSL 3734 22 May 1998	Sea lion faeces	1.31 μg ml^{-1}	
CSL 3758 23 May 1998	Sea lion faeces	96.08 μg ml^{-1}	

Note the range of DA equivalent concentrations found in samples testing positive for that toxin. One sample representing each sample type was also screened for DA using LC-MS/MS. The amount of DA activity found in all phytoplankton samples is plotted in Fig. 1b; results listed here are for samples dominated by *P. australis* only. A complete list of sea lion samples examined with corresponding results of DA analyses is given in ref. 17.

* SCW, phytoplankton samples from Santa Cruz Wharf; C1, phytoplankton samples from station C1 (Fig. 2a); anchovy, anchovies harvested from Monterey Bay, stomachs dissected; CSL, California sea lion, The Marine Mammal Center identification number[17].

† Concentration of DA equivalent to a certified reference standard[12]; concentrations for phytoplankton were calculated based on the number of *P. australis* cells in a sample as estimated by whole cell[9] and sandwich[10,11] hybridization.

‡ Positive, confirmation of the presence of DA without quantification; nd, not detected; unexposed control samples of each matrix type were shown to be negative.

freshwater inputs have been noted elsewhere[15]. Most estimates of cellular toxin levels ranged between ~7–32 pg DA equivalents per *P. australis* cell, similar to that reported previously[16]. However, one sample was estimated to have as much as ~75 pg DA equivalents per *P. australis* cell (Table 1, Fig. 2b). A similarly high per cell value of DA associated with a natural population of *P. australis* in California coastal waters was also recorded in 1998 (V.T. *et al.*, unpublished observation). As the *P. australis* bloom declined, a variety of other *Pseudo-nitzschia* species flourished. *Pseudo-nitzschia pseudodelicatissima* was especially abundant, reaching a maximum density of ~10^6 cells l^{-1} (Fig. 1c, mid-June 1998). During this transition in species composition, DA activity declined precipitously and became undetectable (Fig. 1c).

Blue mussels (*Mytilus edulus*) collected from Santa Cruz during the *P. australis* bloom and tested using the currently accepted high-performance liquid chromatography–ultraviolet (HPLC–UV) regulatory method[1], contained no DA or only trace quantities (0–2 μg g^{-1}; S.L. and G. Langlois, personal communication) and never exceeded levels considered hazardous for human consumption (⩾20 μg g^{-1})[1]. In sharp contrast to mussels, anchovies and sardines caught from Monterey Bay during the *P. australis* bloom and analysed by HPLC–UV contained much more toxin (~30–110 μg g^{-1}; S.L. and G. Langlois, personal communication). The rise and fall of DA in these fish corresponded to the appearance and disappearance of *P. australis* (Fig. 1a). Stomachs of anchovies collected at the peak of the bloom (~22 May 1998) contained high levels of DA activity (Table 1) as well as very high numbers of *P. australis* frustules, but not frustules of other *Pseudo-nitzschia* species (data not shown). Stomach contents of anchovies caught in Monterey Bay also reflected the marked decline of *P. australis* and sudden rise of *P. pseudodelicatissima* (data not shown), along with the corresponding drop in DA activity (4 June 1998; Table 1).

The number of bird and marine mammal carcasses found along the beaches of Monterey Bay increased markedly during the *P. australis* bloom[17]. The most frequently encountered marine mammal was the California sea lion, and live animals of this species were reported as suffering neurological dysfunction. Although over 400 sea lion carcasses were observed between 18 May and 19 June 1998, only 70 live sea lions and one northern fur seal (*Callorhinus ursinus*) were examined clinically and histopathologically at The Marine Mammal Center (TMMC). Of those 70 animals, 28 were found along the beaches of Monterey Bay; of those 28 animals, 20 were collected between 24 and 30 May (ref. 17), a time when the *P. australis* cell density and DA activity had peaked. Reports of live, sick sea lions in Monterey Bay decreased sharply from the end of May onwards, and ceased altogether after 5 June, coinciding with the observed decline in *P. australis* cell density and DA activity (Fig. 1a, b).

Of the 70 animals cared for by TMMC, 48 died despite treatment. Those that recovered appeared clinically normal. All affected animals displayed similar neurological symptoms including seizures, head weaving, ataxia, depression and abnormal scratching[17], the latter being reminiscent of the scratching behaviour documented in mice and pelicans poisoned with DA[1]. The animals were also in good body condition and had haematological and serum biochemical parameters within normal limits[17]. Histological examination of tissues from all 48 sea lions that died revealed unique lesions in the brain and heart. Brain lesions, characterized by zonal vacuolation of the hippocampal neuropile involving several architectural strata, were most severe in the anterior ventral hippocampus (Fig. 3). Such lesions are similar to those observed in mice, macaques and humans exposed to DA[18–20].

Sea lion urine, serum or faeces from 48 animals were tested for DA by receptor-binding assay, with selected samples analysed by LC-MS/MS (Table 1). Receptor-based toxin activity was detected in each sample type, but not from all animals examined[17]. Results of LC-MS/MS analyses established the presence of DA in sea lion urine and faeces, and represent the first unequivocal localization of this toxin in mammalian body fluids in connection with a DA-implicated mortality event. Faecal samples from two sea lions in which DA was detected also contained *P. australis* frustules (K.L., unpublished observation), providing an additional link between sickened animals and the DA-producing diatom.

Unambiguous confirmation of DA in samples of *P. australis*-dominated phytoplankton, northern anchovy and sea lion body fluids provides evidence for the trophic transfer of this toxin from its algal source to a marine mammal via a fish vector. This conclusion is strengthened further by the temporal association of toxic *P. australis* with this mortality event (Fig. 1a and b), the fact that anchovies are a well known prey of sea lions[21], the finding of *P. australis* frustules and anchovy vertebrae in some faecal samples of sickened sea lions[17], detection of DA in body fluids of some sea lions suffering neurological dysfunction, and brain lesions in dead sea lions consistent with exposure to harmful levels of DA (Fig. 3). The total number of sea lions that may have suffered from DA poisoning is not known, but is certainly much greater than the 70 individuals brought to TMMC. Much of the coast affected by the bloom is inaccessible or infrequently visited, counts of dead animals were restricted to specific beaches, no comprehensive land or sea-based surveys for affected animals were undertaken, and only live stranded individuals reported to and retrieved by TMMC were examined. Consequently, the full impact of this bloom on the sea lion population is difficult to gauge.

Although this report provides the first conclusive evidence linking sea lion deaths to a documented HAB, events similar to those described herein appear to have occurred previously. For example, Ocha *et al.*[22] have described dolphin and sea lion mortality associated with *Pseudo-nitzschia* blooms in Mexico, but the cause of death was not established conclusively. Along the California coast, adult sea lions and northern fur seals suffering from neurological dysfunction similar to that documented here were also noted in 1978, 1986, 1988 and 1992 (ref. 17). Animals that died in 1992

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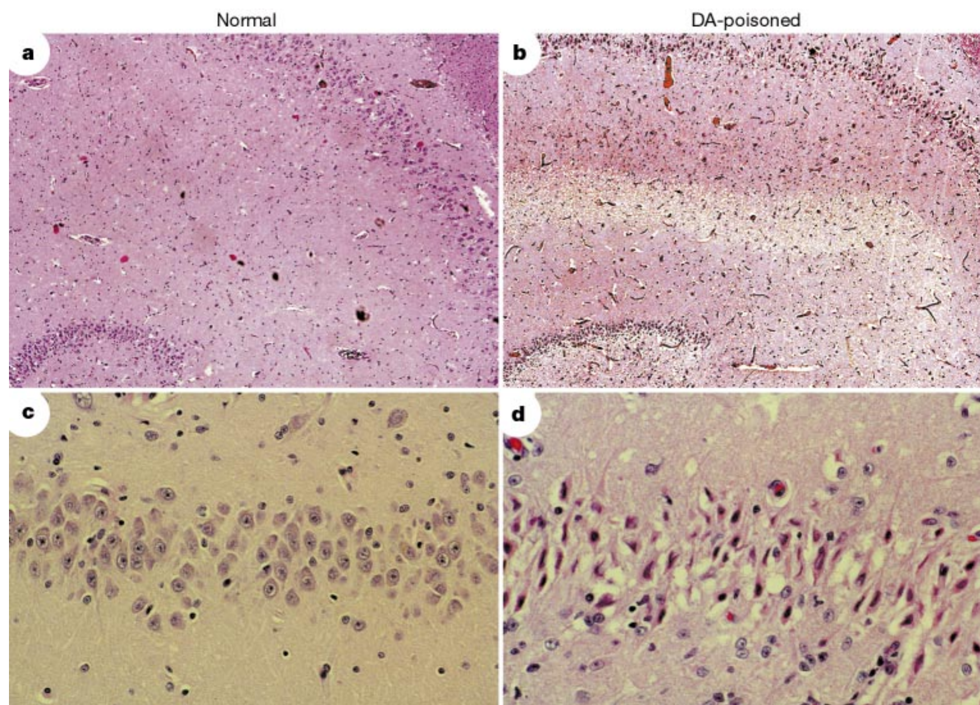

Figure 3 Histological sections of the anterior ventral hippocampus from normal and DA-poisoned California sea lions. Sections prepared with haematoxyalin and eosin[27]. **a**, Anterior ventral hippocampus from a normal animal (original magnification 13.2×). **b**, Section as in **a**, from a DA-poisoned animal, showing pronounced laminar pallor due to vacuolation involving several strata (radiatum, lauculosum and moleculare of both the hippocampus and dentate gyrus). **c**, Close-up of dentate gyrus from a normal animal (original magnification 100×). **d**, Section as in **c**, from a DA-poisoned animal showing acute neuronal necrosis and vacuolation.

displayed brain lesions characteristic of DA poisoning, although the connection to this toxin was not established at that time. Toxic blooms of *Pseudo-nitzschia* can be expected in the future along the shores of California and elsewhere. The rapid and dramatic nature of the 1998 mortality event suggests that even a short 'pulse' of DA in the food web, lasting only days or weeks in a localized area, may be sufficient to kill or sicken marine wildlife. □

Methods

Plankton analysis and nutrient determinations

Whole-water (unconcentrated) plankton samples were collected from the surface at locations shown in Fig. 2a and were subjected to DNA probe-based tests using whole-cell[9] and sandwich[10,11] hybridization techniques to identify and quantify a variety of toxic and nontoxic *Pseudo-nitzschia* species. Reagents and instrumentation needed for the sandwich hybridization assay are available commercially (Saigene). Aliquots of the water samples were also subjected to DA[12] and nutrient[23] analyses. Data shown here are for the SCW station only.

Domoic acid analysis

Particulate material in whole-water field samples (150–750 ml) was collected onto 25 mm GF/F filters (Whatman), then frozen at −70 °C. Filters were extracted in 10% aqueous methanol and tested using a DA receptor binding assay[12]. The following modifications were implemented: concentrations and volumes of reagents used in the glutamate decarboxylase (GAD) digestion to eliminate ambient glutamate from extracts were adjusted to provide a two-fold sample dilution; assays were performed in 96-well MultiScreen plates (Millipore), after which 25 μl of Optiphase liquid scintillant were added to each well and plates counted directly on a microplate scintillation counter (Microbeta 1450, Wallac, Inc.). The DACS-1B certified DA reference standard (National Research Council, Halifax) was used to calibrate the assay and determine equivalent DA activity in a sample. Values are expressed as pmol DA equivalents per ml whole water filtered and indicate DA activity in the ⩾0.7 μm fraction normalized to volume filtered.

Detection of DA activity in sea lion faeces and anchovy samples by receptor binding assay required an initial extraction with 50% aqueous methanol[24]. Faecal extracts (only) were further cleaned using a strong anion exchange (SAX) solid-phase extraction protocol[25]. Anchovy and cleaned faecal extracts, as well as native sea lion serum and urine samples, were subjected to a GAD digest and run on the receptor assay as described above. The sample of each type showing the highest DA concentration was then analysed by LC-MS/MS to confirm the presence of the toxin. The same extracts or native samples (without GAD digestion) were fractionated on a C18 column using a gradient of 1–95% methanol in 0.1% TFA. A PE SCIEX API-III triple quadrupole mass spectrometer was employed, with the ionspray source operated in positive ion mode, using compressed air as the nebulization gas. The first quadrupole was used to pass only ions of nominal 312 *m/z*. The second quadrupole was used to facilitate a substantial amount of collisionally induced dissociation, while the third quadrupole was operated in multiple ion monitoring mode where fragment ions of 161 and 266 *m/z*, as well as residual parent ions (312 *m/z*), were allowed to pass to the ion detector.

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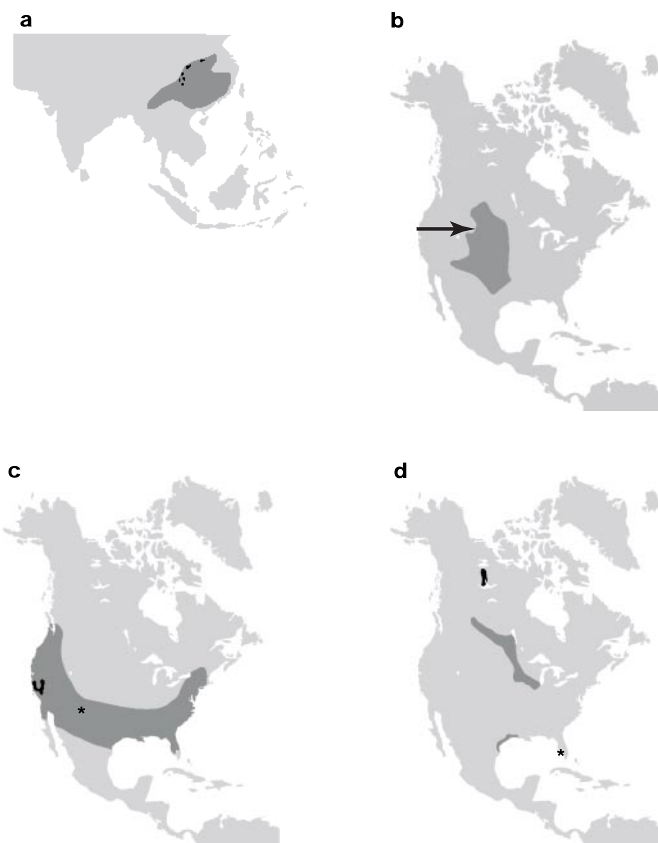

Figure 1 Patterns of range contraction in four endangered species. **a**, Giant panda, *Ailuropoda melanoleuca*; **b**, black-footed ferret, *Mustela nigripes*; **c**, California condor, *Gymnogyps californianus*; **d**, whooping crane, *Grus americana*. Historical range is in grey, extant range is in black or indicated by an arrow, and asterisks mark the locations of recent re-introduction sites for the California condor and the whooping crane.

Dynamic biogeography and conservation of endangered species

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As one moves from the core to the periphery of a species' geographical range, populations occupy less favourable habitats and exhibit lower and more variable densities[1–4]. Populations along the periphery of the range tend to be more fragmented and, as a result, are less likely to receive immigrants from other populations. A population's probability of extinction is directly correlated with its variability and inversely correlated with density and immigration rate[5–9]. This has led to the prediction that, when a species becomes endangered, its geographical range should contract inwards, with the core populations persisting until the final stages of decline[2,10]. Convinced by these logical but untested deductions, conservation biologists and wildlife managers have been instructed to avoid the range periphery when planning conservation strategies or allocating resources for endangered species[11–13]. We have analysed range contraction in 245 species from a broad range of taxonomic groups and geographical regions. Here we report that observed patterns of

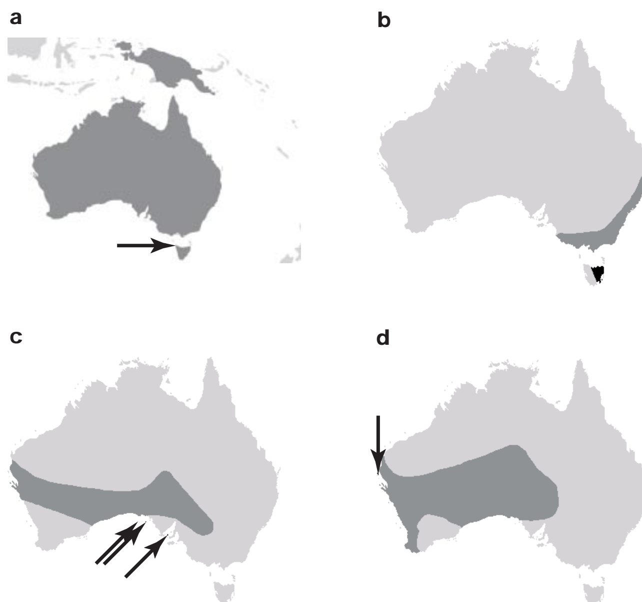

Figure 2 Patterns of range contraction in four species whose historical range included islands as well as much larger areas on the Australian mainland. **a**, Tasmanian tiger, *Thylacinus cynocephalus*; **b**, Tasmanian bettong, *Bettongia gaimardi*; **c**, greater stick-nest rat, *Leporillus conditor*, **d**, Shark Bay mouse, *Pseudomys fieldi*. Historical range in grey, and extant or final range is in black or indicated by an arrow.

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