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Too much of a good thing; sea ice extent may have forced emperor penguins into refugia during the last glacial maximum

Running head: Emperor penguins forced into glacial refugia.

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Abstract

1   The relationship between population structure and demographic history is critical to

2   understanding microevolution and for predicting the resilience of species to

3   environmental change. Using mitochondrial DNA from extant colonies and radiocarbon-

4   dated subfossils, we present the first microevolutionary analysis of emperor penguins

5   (*Aptenodytes forsteri*) and show their population trends throughout the last glacial

6   maximum (LGM, 19.5 – 16 kya) and during the subsequent period of warming and sea

7   ice retreat. We found evidence for three mitochondrial clades within emperor penguins,

8   suggesting that they were isolated within three glacial refugia during the LGM. One of

9   these clades has remained largely isolated within the Ross Sea, while the two other clades

10   have intermixed around the coast of Antarctica from Adélie Land to the Weddell Sea. The

11   differentiation of the Ross Sea population has been preserved despite rapid population

12   growth and opportunities for migration. Low effective population sizes during the LGM,

13   followed by a rapid expansion around the beginning of the Holocene, suggest that an

14   optimum set of sea ice conditions exist for emperor penguins, corresponding to available

15   foraging area.

1   **Introduction**

2   Genetic data both from modern and subfossil samples, palaeo-ecological niche modeling,

3   and fossil evidence have become vital tools for reconstructing demographic histories (e.g.

4   woolly mammoths (*Mammuthus primigenius*) (Nogués-Bravo *et al.*, 2008) and lions

5   (*Panthera leo*) (Barnett *et al.*, 2014)). Indeed such studies have shown that species'

6   patterns of genetic diversity and distribution have varied dramatically under different

7   climatic regimes (Carstens & Richards, 2007). Climatic shifts have been one of the major

8   drivers of species' range shifts, fluctuations in abundance, species extinctions and also in

9   the formation of genetically distinct populations (Hewitt, 1996). As climate change and

10   habitat degradation potentially take us into the 6th mass extinction (Barnosky *et al.*, 2011),

11   it is critical that we understand how species have coped with change in the past to be able

12   to assess their likely responses and resilience to future climate change (Hoelzel, 2010).

13   Emperor penguins (*Aptenodytes forsteri*) are an iconic Antarctic species whose

14   population genetic structure has not been studied to date. We know little about dispersal

15   among colonies or how historical climate change may have affected their range and

16   abundance. Thus, we have limited capacity to predict how these birds may fare in the

17   future. Projections for continent-wide declines of emperor penguins have been made

18   based on the demographic responses of the Pointe Géologie colony to changes in sea ice

19   conditions (Barbraud & Weimerskirch 2001, Jenouvrier *et al.*, 2009, Ainley *et al.*, 2010,

20   Jenouvrier *et al.*, 2012, Jenouvrier *et al.*, 2014). However, decadal monitoring data is only

21   available for this single site out of 46 known emperor penguin colonies; as such, the

22   climate change responses of emperor penguins across their entire distribution and over

23   millennial timescales are currently unknown (Ainley *et al.*, 2010).

1   Emperor penguins are highly reliant on sea ice throughout most of their breeding cycle,

2   and mating and incubation takes place on land-fast sea ice in most of the known colonies

3   (Fretwell *et al.*, 2012, Fretwell *et al.*, 2014). During the breeding season, emperor

4   penguins feed on prey that is also sea ice dependent (Gales *et al.*, 1990). Significant areas

5   of open water exist year-round within the Antarctic sea ice zone in the form of leads and

6   polynyas (Zwally *et al*,. 1985 and references therein). These areas are often important in

7   providing emperor penguins access to their underwater foraging habitat when the fast ice

8   extends far from their colonies (Dewasmes *et al*., 1980). Polynya formation is driven by

9   either upwelling of Circumpolar Deep Water or by the outflow of katabatic winds that

10   push sea ice away from the coastline (Martin, 2001). Polynyas are associated with

11   enhanced primary production, as the reduction in sea ice volume facilitates an earlier

12   spring melting of sea ice and a coincident earlier start in photosynthetic primary

13   productivity (Martin, 2001). Some polynyas are permanent features of the sea ice zone and

14   create areas of hyperproductivity, such as the Ross Sea polynya (Smith & Gordon, 1997),

15   whilst most are smaller and ephemeral features depending on wind stresses and currents.

16   Changes in the extent and duration of sea ice around Antarctica show highly regionalized

17   trends with some areas increasing or remaining stable while others are decreasing

18   (Vaughan *et al.*, 2013, Zwally *et al.*, 2002); this has an effect on the population dynamics

19   of emperor penguins as both positive and negative sea ice anomalies can result in

20   negative population growth rates at the local scale (Massom *et al.*, 2009, Ainley *et al*,.

21   2010, Barbraud *et al*., 2011, Jenouvrier *et al*., 2014). Despite uncertainties over the rate

22   and extent of ice loss that will occur around Antarctica, all climate models project a

23   reduction in the extent and duration of Antarctic sea ice by the end of the century (Collins

24   *et al.*, 2013). As sea ice declines we might expect emperor penguins to be disadvantaged

25   by a lack of breeding habitat (Jenouvrier *et al.*, 2014), unless they have the capacity to

alter their preferred choice of breeding site or their range by colonising new areas. Recent studies have shown more plasticity than expected in the locations of breeding colonies; satellite imagery suggests that colonies where the fast ice is inadequate at the onset of the breeding season relocated or partially relocated onto ice shelves or ice-bergs (Fretwell *et al.*, 2014, LaRue *et al.*, 2014). However, as sea ice declines, emperor penguins may also have to contend with altered prey availability and face new threats from predators as changing conditions differentially affect species at other trophic levels (Trathan *et al.*, 2011).

During the last glacial maximum (LGM, 19.5 – 16 kya), the winter sea ice extent was approximately double the present day values, and seasonal variation in sea ice extent is thought to have been greater (Gersonde *et al.*, 2005). It is unclear how this would have affected emperor penguins. Thatje and colleagues (2008) suggested that they may have migrated with the sea ice to lower latitudes, staying within energetic migration thresholds of the ice edge, and could have maintained breeding populations around Antarctica by foraging in the marginal ice zone at the sea ice edge. Alternatively, they could have remained associated with polynyas. Sediment cores suggest the existence of LGM polynyas in several locations, including the north-western Ross Sea, the south-eastern Weddell Sea off Dronning Maud Land, and the north-western Weddell Sea (Mackensen *et al*., 1994, Brambati *et al*., 2002, Thatje *et al*., 2008, Smith *et al*., 2010). In either case, reductions in overall primary productivity within what is today's seasonal sea ice zone (Domack *et al*., 1998, Kohfeld *et al*., 2005) would likely have been detrimental to emperor penguin populations (Ainley *et al,*. 2010).

Little is known about the level of natal philopatry or migration among emperor penguin colonies. Understanding philopatry is particularly important in light of population models that suggest that emperor penguins may be declining as a result of local climatic shifts

(Jenouvrier *et al.*, 2009, Jenouvrier *et al.*, 2014). High emigration rates are conceivable amongst emperor penguin colonies; satellite tracking has shown that they travel thousands of kilometres on their juvenile journeys, often passing other colonies (e.g. Kooyman *et al.*, 1996, Thiebot *et al.*, 2013, Wienecke *et al.*, 2010). Generally, philopatry is high amongst penguins (Dehnhard *et al.*, 2014, Saraux *et al.*, 2011), but population structure is absent in many species (e.g. Chinstrap penguins (*Pygoscelis antarctica*) (Clucas *et al.*, 2014)) as even low levels of migration can be sufficient to homogenize populations (Hartl & Clark, 1997).

We analyzed the population structure among eight extant emperor penguin colonies (Figure 1) using mitochondrial DNA sequences, and inferred population trajectories during and since the LGM using a combination of ancient and modern DNA sequences in a Bayesian coalescent framework (Drummond *et al.*, 2005). This method reconstructs past changes in abundance by estimating the genealogy from sequence data, and co-estimating the effective population size at different points in time, where the effective population size is the number of individuals that contribute offspring to the descendant generation (Pybus *et al.*, 2000). We aimed to: 1) investigate how emperor penguin populations were affected by sea ice conditions during and following the LGM; and 2) to test the hypothesis that emperor penguins comprise one panmictic population as a result of the high dispersal of individuals after fledging, and the lack of obvious ecological barriers to dispersal around the Antarctic coastline.

1	**Materials and methods**
2	*Sample collection*
3	Skin tissue of dead emperor penguins was collected from Halley Bay (see Figure 1 for all
4	sample locations) in November 2012 and transported frozen to the UK, where it was
5	transferred to 90% ethanol and stored at -20°C. Blood samples were collected from Gould
6	Bay in December 2013 and transported to the UK at ambient temperature in RNAlater
7	(Life Technologies), and then stored at -20°C. Shed feathers were collected from the Ross
8	Sea between 2010 and 2012, and were transported and stored at -20°C. Shed feathers
9	were collected at least 10 meters apart to minimize sampling the same bird. Pectoral
10	muscle biopsies were collected from dead chicks at Fold Island in September 2010, from
11	Pointe Géologie in December 2010 and from Amanda Bay in December 2012 and 2013.
12	Biopsies were immediately placed in 90% ethanol and stored at -20°C. Whole dead
13	chicks were collected from Auster in September and October in 1993 and 1994 and
14	transported and stored at -20°C. Bones from the subfossil remains of three penguins were
15	collected at Club Lake in January 2013 and stored at -80°C. Club Lake is an ice-free area
16	in the Vestfold Hills which is currently unoccupied by penguins. The nearest extant
17	colony is Amanda Bay, 95 km away.
18	Where blood samples were taken, one handler seized the upper body with both hands and
19	restrained the flippers, with the bird's head placed under the arm of the handler to prevent
20	biting and minimize stress (Le Maho *et al.*, 1992). The second handler took blood from
21	the brachial vein using a 25G needle and 1 mL syringe. Total restraint time was generally
22	two to three, but occasionally four, minutes. The bird was then released at the edge of the
23	colony. Sampling was conducted under permits from the UK Foreign and Commonwealth
24	Office, the US National Science Foundation and the Australian Antarctic Division. Each
25	of these permits was issued following independent ethical review of the sampling. All

sampling was carried out in accordance with UK Home Office guidelines and also

received ethical approval from the University of Oxford, British Antarctic Survey and

Australian Antarctic Division. The radiocarbon ages, expressed here as years BP (i.e.

before 1950), of the Club Lake remains were determined using accelerated mass

spectrometry by GNS Science Rafter Radiocarbon National Isotope Centre, New Zealand.

The apparent ages were corrected for the marine-carbon reservoir effect (Gordon &

Harkness, 1992) using the calibration program Calib7.0 (ST UI & Reimer, 1993).

DNA extraction, amplification and sequencing

Genomic DNA (gDNA) was extracted from modern samples with the QIAGEN DNeasy

Blood and Tissue Kit. The manufacturer's protocols for blood and tissue samples were

followed with the following modifications to the digestion step: for blood samples 30 μL

of proteinase-K was used and the digestion time was 3 h; for tissue samples 40 μL

proteinase-K and an additional 10 μL 1 M dithiothreitol (skin samples only) was used

with an incubation time of 32 h. All samples were treated with either 1 μL RNase A

(QIAGEN) or 1 μL Riboshredder (Epicentre) according to the manufacturers' instructions.

DNA was eluted in 100 μL of elution buffer following an incubation of 5 – 20 min. For

subfossil samples ~50 mg of bone was decalcified in 0.5 M EDTA/0.001% Triton X100

at 56°C for 48 h and then extracted using a standard phenol chloroform protocol with

ethanol precipitation and a final elution volume of 30 μL. The subfossil samples were

extracted in a physically isolated laboratory which had not been used previously for avian

samples to minimise the risk of contamination. The mitochondrial hypervariable region

(HVR) and cytochrome B (CytB) were sequenced in all modern and ancient DNA

samples. HVR is a rapidly evolving region of the mitochondrial genome, and so is

suitable for investigations of recent demographic history, whilst CytB is a conserved gene

and can hence give information about longer-term demographic history (Baker &

Marshall, 1997). HVR was amplified in all modern samples using primers F-0225 and R-INR (all primer sequences can be found in Supplementary Table 1). The reaction mix consisted of 7.5 µL of PCR Master Mix (QIAGEN), 0.2 µM of each primer, and 5 -10 ng gDNA, made up to 15 µL with ddH$_2$O. Thermocycling conditions were: 94°C for 3 min; 35 cycles of 94°C for 30 s, 59.5°C for 45 s, 72°C for 1 min; followed by an extension period of 72°C for 10 min. Occasionally, double bands were apparent when the PCR product was visualized by electrophoresis. For these individuals, the shorter 755 bp band was extracted from the gel and purified using QIAGEN or Promega gel extraction kits following the manufacturer's instructions. For Fold Island, Amanda Bay, Auster and Pointe Géologie colonies, CytB was amplified using primers B1 (Baker *et al.*, 2006, Kocher *et al.*, 1989) and B6 (Baker *et al.*, 2006) with a reaction mix consisting of 7.5 µL of GoTaq Green Master Mix (Promega), 0.2 µM of each primer, and 5-10 ng gDNA, made up to 15 µL with ddH$_2$O. Thermocycling conditions were: 95°C for 1 min; 35 cycles of 95°C for 20 s, 52°C for 40 s, 72°C for 50 s; then 72°C for 5 min. For the Cape Washington, Cape Crozier, Gould Bay and Halley Bay samples, primers CytB-F1 and CytB-R1 were used with a reaction mix consisting of 7.5 µL of PCR Master Mix (QIAGEN), 0.2 µM of each primer, and 5 -10 ng gDNA, made up to 15 µL with ddH$_2$O. Thermocycling conditions were: 94°C for 3 mins; 35 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 1 min; then 72°C for 10 min. For the subfossil samples we designed novel, species-specific primers (Supplementary Table 1) to amplify short (<150bp) overlapping fragments in order to improve the success rate of amplification from degraded DNA. The reaction mix consisted of 7.5 µL of AmpliTaq Gold 360 Master Mix (Life Technologies), 0.2 µM of each primer, and 25-50 ng gDNA, made up to 15 µL with ddH$_2$O. Thermocycling conditions were: 95°C for 10 min; 42 cycles of 95°C for 20 s, T$_m$ (primer) for 20 s, 72°C for 20 s; 72°C for 5 min. PCR products for Fold Island, Amanda Bay,

Auster, Pointe Géologie and the subfossil samples were bi-directionally sequenced by the Australian Genome Research Facility (AGRF) via the Sanger sequencing method using the PCR primer pairs. PCR products for Gould Bay, Halley Bay, Cape Washington and Cape Crozier were sequenced using the Sanger method by Macrogen Europe. The reverse primer for the HVR and the forward primer for CytB were used to sequence each product twice, as these were found to work best in the sequencing reaction. Geneious v5.5.9 was used for alignment. A high number of heteroplasmic sites were found in the HVR and these were re-scored manually according to IUPAC ambiguity codes. No heteroplasmic sites were recorded in the CytB sequences.

Data analysis – summary statistics and population structure

Arlequin v3.5 (Excoffier & Lischer, 2010) was used to calculate summary statistics for HVR, CytB, and concatenated HVR and CytB. jModeltest (Posada & Buckley, 2004) was used to estimate the best substitution model for each dataset, and then the following corrections for calculating genetic distances were implemented in Arlequin: HVR - Tamura correction with a gamma distribution for rate heterogeneity with $\alpha = 0.016$; CytB – Tamura correction; concatenated – Tamura correction with a gamma distribution for rate heterogeneity with $\alpha = 0.109$ (Tamura, 1992). Arlequin was also used to calculate pairwise genetic distances (θ_{ST}) between colonies and perform analyses of molecular variation (AMOVA) on the concatenated sequences with the Tamura & Nei correction. Network v4.612 (Fluxus Technology Ltd.) was used to draw haplotype networks.

Data analysis - demographic histories

Bayesian phylogenetic analyses and demographic reconstructions were performed using BEAST v1.8 (Drummond et al., 2012). The dataset was partitioned into HVR and CytB, with a nucleotide substitution model of HKY (Hasegawa et al., 1985) with four gamma categories for HVR and TN93 (Tamura & Nei, 1993) for CytB, with ambiguous states

permitted. We used the coalescent Extended Bayesian Skyline Plot tree prior (Heled & Drummond, 2008) with a strict molecular clock. For molecular clock calibration, the HVR substitution rate prior was specified as a normal distribution around a mean value of 0.55 substitutions/site/Myr (SD = 0.15), to reflect the substitution rate of the HVR in Adélie penguins (*Pygoscelis adeliae*) (Millar *et al.*, 2008). In the absence of a published substitution rate for CytB in penguins we used a uniform prior of 5×10^{-4} to 5×10^{-1} substitutions/site/Myr with a starting value of 2×10^{-2} (Weir & Schluter, 2008). The corrected radiocarbon ages of the Club Lake samples were input as tip dates, for additional calibration of the molecular clock. Based on these initial priors, substitution rates for our dataset were estimated during the analysis. The posterior distributions of substitution rates, phylogenetic trees and effective population size through time were generated using the Markov chain Monte Carlo (MCMC) sampling procedure, implemented in BEAST, which was run for 120 million generations with samples drawn every 6000 steps and the first 10% discarded as burn-in. Tracer v.1.5 was used to check effective sample size (ESS) values to confirm convergence with all values >200. Three independent BEAST analyses were performed to ensure reproducibility of the posterior distribution. The population size parameter of the demographic model (N_e*tau) was converted to N_{ef} by dividing the parameter by 14 years, which is the estimated generation length of emperor penguins (Forcada & Trathan, 2009, Jenouvrier *et al.*, 2005). Phylogenetic trees were visualised using FigTree v1.4.

Results

Present day population structure

1 We sequenced 226 individuals from eight colonies (Figure 1) plus three subfossil birds whose ages ranged from 643 – 881 years BP (after correction for marine reservoir effect). We sequenced 629 bp of the mitochondrial hypervariable region (HVR) and 867 bp of cytochrome *b* (CytB) from each individual (GenBank accession numbers KP644787 - KP645015 and KP640645 - KP640873, respectively). Genetic diversity was extremely high for the HVR, with 220 haplotypes recorded out of the 229 individuals sequenced; the mean number of pairwise differences between haplotypes was 20.62 ± 9.14 (Table 1). Genetic diversity was much lower for CytB, with just 59 unique haplotypes recorded.

Our results show a high level of gene flow among all the EAWS colonies (East Antarctica including Adélie Land, and the Weddell Sea) and between the two Ross Sea colonies (Table 2), but little exchange between the EAWS and Ross Sea colonies (pairwise θ_{ST} values range from 0.213 to 0.617, Table 2). When colonies are grouped into two populations (Ross Sea and EAWS), a high proportion (17.7%) of the genetic variation is explained by the difference between the groups, and there is strong and significant genetic differentiation between them (AMOVA, $F_{ST} = 0.196$, $p<0.001$). This pattern is also evident from haplotype networks (Supplementary Figures 1 & 2), which show that Ross Sea individuals tend to be closely related, whilst sequences from EAWS colonies tend to cluster independently from the Ross Sea haplotypes. However, some Ross Sea sequences are found across the network, and vice versa. This could indicate low-level gene flow between the Ross Sea and EAWS.

Population history with respect to climate change

There is evidence of past population expansion in emperor penguins across Antarctica as indicated by our Extended Bayesian Skyline Plots (EBSPs) (Figure 2). An almost nine-fold increase in abundance of the EAWS population commenced approximately 12 kya. The Ross Sea population expanded three-fold from approximately 9.5 kya. Superimposing expansion signals over the estimated temperature derived from ice cores (Figure 2c), it is clear that population expansion followed the end of the LGM. Tajima's *D* and Fu's F_S statistics provide further support for an expansion of both populations (Table 1).

Our phylogenetic analyses indicate three highly supported clades (Figure 3), which diverged during the Late Pleistocene (97 kya, 95% HPD: 50−154 kya). One of these clades is comprised predominantly of Ross Sea penguins, whereas the other two are dominated by EAWS individuals.

Discussion

This first analysis of emperor penguin population structure shows colonies within the Ross Sea are genetically distinct from other Antarctic colonies, whereas those from the rest of the continent and spanning up to 8000 km of coastline are panmictic (Table 2). The admixture of the EAWS emperor penguins supports our hypothesis of limited population structure and indicates a very large dispersal range for the species. Given our genetic evidence of extensive mixing across Antarctica, the unique structure in the Ross Sea emperor penguins is surprising, and interestingly the same pattern was reported for the sympatric Adélie penguin (Ritchie *et al.*, 2004), providing further evidence that the Ross Sea has a unique evolutionary history.

The existence of distinct penguin populations in the Ross Sea is puzzling. There are neither geographic nor oceanographic barriers isolating the Ross Sea from the rest of Antarctica. Furthermore, the relative distance between the Ross Sea and other colonies does not adequately explain its isolation as, for example, the Pointe Géologie colony is approximately 5600 km closer to the Ross Sea colonies than to those in the Weddell Sea (Figure 1). Emperor penguins are known for their extraordinary migrations; satellite tracking showed that juveniles can travel >7000 km in eight months (Thiebot *et al.*, 2013). These observations support our genetic results for the EAWS region and indicate juvenile emperor penguins could comfortably traverse the 1800 km between Pointe Géologie and the Ross Sea colonies. There are also no clear habitat, environmental or foraging differences between the Ross Sea colonies and those located elsewhere (Budd, 1961, Smith *et al.*, 2012), except that Ross Sea colonies are located closer to the ice edge, and are therefore potentially more resilient to increases in sea ice. We suggest that the divergence of emperor penguins into two populations is historical in origin.

There are three ancestral lineages within modern emperor penguins, providing evidence

that populations were isolated in the past (Figure 3) and diverged through

microevolutionary processes, such as selection or genetic drift, which occur more rapidly

in small, isolated populations (Hewitt, 2000). One of these lineages is mostly limited to

the Ross Sea, indicating that the isolation of this region has persisted through time.

Indeed, emperor penguins occupying the Ross Sea may have become so differentiated

that interbreeding with the EAWS penguins occurs at very low rates, perhaps because of

genetic, behavioural (Templeton, 1981) or cultural incompatibilities, such as the timing of

breeding or the development of regional dialects (de Dinechin *et al.*, 2012, Jouventin &

Aubin, 2002, MacDougall-Shackleton & MacDougall-Shackleton, 2001).

Emperor penguins use complex display calls to recognise their mates and offspring

(Robisson *et al.*, 1993). Vocalisation is known to be an important part of the courtship

process for most penguins (Richdale, 1944, Waas *et al.*, 2000). Interestingly, royal

penguins (*Eudyptes schlegeli*) respond more strongly to calls from their own colony

members than to calls originating from different colonies, suggesting differences in

dialect (Waas *et al.*, 2000). Differences in vocalisations have also been found among

gentoo penguin (*Pygoscelis papua*) populations (de Dinechin *et al.*, 2012). If dialects

become too different, then courtship may be inhibited, thereby limiting interbreeding.

This has been observed in passerine birds, in which genetically distinct groups have

unique mating songs (MacDougall-Shackleton & MacDougall-Shackleton, 2001).

Emperor penguin vocalisation patterns have only been recorded at Pointe Géologie

(Robisson *et al.*, 1993), but our hypothesis could be explored in the future by comparing

vocalisations of emperor penguins from the Ross Sea with those of other colonies.

Although the isolation and differentiation of the Ross Sea emperor penguins has persisted,

the other two historical lineages show no geographic bias and have now hybridized to

form one EAWS population. Incomplete mixing of ancestral lineages is typical of species that have survived the Pleistocene ice-ages in multiple refugia (Hewitt, 1996). Our EBSPs indicate that both the EAWS and Ross Sea populations had reduced effective population sizes during the LGM (Figure 2). Thus, contrary to a hypothesis that emperor penguins would benefit from glaciation as a result of reduced competition with other predators (Thatje *et al.*, 2008), it seems that they, like other Antarctic and sub-Antarctic penguin species (Clucas *et al.*, 2014, Ritchie *et al.*, 2004, Trucchi *et al.*, 2014), were adversely affected by the LGM.

We propose that both the reduced abundance and divergence into three lineages were linked to breeding and foraging habitat availability. Today emperor penguins have a circumpolar distribution with suitable habitat spanning the entire continent (Fretwell & Trathan, 2009). However, Antarctica during the LGM looked very different than the continent we know today (Figure 4). Most of the continental shelf was covered by ice as a result of both the extension of ice-sheets and thick, perennial sea ice, which reduced productivity south of the modern-day Polar Front drastically (Anderson *et al.*, 2002, Anderson *et al.*, 2009, Domack *et al.*, 1998, Gersonde *et al.*, 2005, Kohfeld *et al.*, 2005, Samuel Jaccard pers. comm.). We suggest that the increased sea ice extent would have severely restricted the foraging habitat available for emperor penguins and, coupled with lower primary production, could have resulted in a scarcity of prey resources. Additionally, air temperatures were approximately 13°C colder than the present day (Jouzel *et al.*, 2007), which may have been near the penguins' lower limit of temperature tolerance (Le Maho *et al.*, 1978), potentially impacting both breeding success and adult survival.

The extent and duration of sea ice are important factors in the breeding success of emperor penguins (Massom *et al.*, 2009). Emperor penguins require stable fast ice to

breed, but they have to traverse the sea ice to establish colonies in autumn and to forage in winter and spring. The distances between the colonies and potential foraging areas can influence breeding success where the fast ice extent is variable (Massom *et al.*, 2009), but not in locations where the extent is relatively stable (Robertson *et al.*, 2013). We therefore expect that if the winter sea ice extent was substantially greater in the LGM, or if the timing of sea ice retreat was altered, that this would have made some of the extant colony locations energetically untenable during the LGM.

During the LGM, the summer sea ice extent was similar to what we observe today, whereas the winter extent was roughly doubled (Gersonde *et al.*, 2005). Colonies may have been located close to the continent so that the ice remained stable throughout the breeding season, but this would have required adults to walk immense distances to reach foraging areas during winter and spring while provisioning the chick. In that case, the chicks would receive fewer meals and be less likely to survive. The present distribution of colonies close to land (Fretwell & Trathan, 2009) suggests that fast ice proximate to land provides a more stable platform than near the fast ice edge. Also, stable ice close to the coast occurs in predictable locations that might be important for colony establishment and cohesion. Colonies further away from the coast may therefore be difficult to maintain. Our discovery of three distinct lineages provides evidence against a straightforward, latitudinal range shift in line with the sea ice edge, and suggests that emperor penguins may have survived the LGM in three suitably situated, geographically isolated refugia.

Emperor penguin refugia during the LGM may have been linked to the presence of polynyas. Several extant emperor penguin colonies are located near polynyas, which may be utilised for foraging during the winter (Croxall *et al.*, 2002). Polynyas acted as "hot spots" of primary productivity during the LGM, supporting marine life and flying seabirds (Thatje *et al.*, 2008). Sediment cores in the north-western Ross Sea indicate open

water polynya conditions throughout the LGM (Brambati *et al.*, 2002, Thatje *et al.*, 2008) and this polynya could have sustained a refuge population until the Ross Sea began to clear of ice (Figure 4). By 9.6 kya most of the northern Ross Sea was open water (Licht & Andrews, 2002). The retreating sea ice and increased upwelling during deglacation increased productivity in the Ross Sea (Anderson *et al.*, 2009) and likely increased the foraging habitat and prey availability to emperor penguins, therefore we hypothesise that these factors drove the three-fold expansion of emperor penguins in this region around this time (Figure 2). The LGM polynya may have also supported Adélie penguins, accounting for the existence of a distinct Ross Sea clade as previously observed for this species (Ritchie *et al.*, 2004).

Another polynya was located in the south-eastern Weddell Sea off Dronning Maud Land (DML) (Mackensen *et al.*, 1994, Thatje *et al.*, 2008) (Figure 4). Colonies of snow petrels (*Pagrodroma nivea*) were present in DML throughout the LGM, associated with this polynya (Wand & Hermichen, 2005), and it may have also provided a refuge for emperor penguins. There is evidence from sediment cores for a third LGM polynya, located in the north-western Weddell Sea (Smith *et al.*, 2010) (Figure 4); this would be consistent with our third emperor penguin refuge, given that the refuge is likely to be more proximate to DML than the Ross Sea, since the two refugial lineages hybridized post-glacially while the Ross Sea lineage remained distinct.

We propose that two refuge populations that were isolated in the Weddell Sea expanded their range into Prydz Bay and Adélie Land and merged during the retreat of the East Antarctic ice sheet 14 – 7 kya (Mackintosh *et al.*, 2011). At this time, the onset of more favourable environmental conditions could have resulted in the dramatic, nine-fold increase in abundance shown here (Figure 2). A seasonal sea ice cycle was established in Prydz Bay approximately 10.4 kya (Barbara *et al.*, 2010), opening up foraging habitat and

coinciding with high levels of primary productivity (e.g. (Anderson *et al.*, 2009, Sedwick *et al.*, 2001). In Adélie Land, primary productivity and the duration of the ice-free season increased from 9 kya (Denis *et al.*, 2009a, Denis *et al.*, 2009b). This new habitat could have facilitated the range expansion of the EAWS lineages.

It should be noted that the timing of the abundance increase of emperor penguins does not coincide exactly with the end of the LGM (Figure 2). We hypothesise that it is not the temperature change itself, but rather the subsequent change in sea ice conditions and primary productivity that are most likely to affect emperor penguins. Indeed, it has been proposed that there is an optimal level of sea ice at the large temporal / spatial scale for emperor penguins, which roughly corresponds to current conditions (Ainley *et al,*. 2010). Therefore, the greater sea ice extent of the LGM was most likely sub-optimal for emperor penguin populations. The end of the LGM is measured when temperatures began to increase (19 – 16 kya). However deglaciation, during which ice-sheets and sea ice retreated and primary productivity increased, occurred slowly over an extended time period (*ca*. 17 – 11 kya) (Anderson *et al.*, 2009). These events occurred later in the Ross Sea than in East Antarctica, and our results support the hypothesis that ice-sheet and sea ice retreat and increasing primary productivity were the main factors controlling emperor penguin abundance, as the Ross Sea emperor penguin population expanded later than the EAWS population (Figure 2). Furthermore, emperor penguins produce only one chick per year and take approximately five years to reach sexual maturity (Jenouvrier *et al.*, 2005), so any abundance increase would be initially slow.

Our hypothesis of three refugial populations of emperor penguins during the LGM could be tested using a higher density of genetic markers. This would allow for the investigation of clinal variation in genetic diversity arising from founder effects as new areas were colonized following the expansion from refugia after the LGM (Hewitt, 1996). It should

be noted that our present study is based on mitochondrial DNA and therefore represents dispersal patterns of females only, but nonetheless supports a plausible explanation for past and present microevolutionary processes in emperor penguins. The next step should be to verify these findings using nuclear markers to account for male-mediated gene flow.

In this continent-wide study of microevolution in an Antarctic penguin we suggest that past climatic changes have greatly impacted emperor penguin populations. As conditions became more favourable after the LGM, their global population expanded and the populations from the Weddell Sea and East Antarctic intermixed to form one large, panmictic population. Interestingly, the isolation of the Ross Sea emperor penguins has persisted until today. The reasons for this isolation remain unknown, but we suggest that separate management plans are required for the Ross Sea and EAWS populations. By conserving the full spectrum of genetic variation and, in particular, all phylogeographic lineages, the evolutionary potential of the species can be maximised (D'Amen *et al.*, 2013).

Our study suggests that emperor penguins have shown important historic responses to past climate shifts and their population increase post-LGM was remarkable. However, the projected rate of temperature increase over the next century is an order of magnitude greater than that following the LGM (Collins *et al.*, 2013, Masson-Delmotte *et al.*, 2013, Shakun *et al.*, 2012). At present, emperor penguins become heat stressed around 0°C, so may exist near the upper limits of their physiological tolerance (Wienecke, pers obs). Whether the resilience demonstrated in the past of this highly cold-adapted species will enable it to adapt to projected climate change remains to be seen, as rising temperatures will alter its breeding grounds and foraging space more rapidly than in the past.

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1   **Supporting Information Legends**

2   **Figure S1. Haplotype network of phylogenetic relationships among all HVR sequences.**
3   Magenta – EAWS colonies; green – Ross Sea colonies; blue – subfossil samples; the size of
4   the circle indicates the relative frequency of the haplotype.

5   **Figure S2. Haplotype network of phylogenetic relationships among all CytB sequences.**
6   Magenta – EAWS colonies; green – Ross Sea colonies; blue – subfossil samples; the size of
7   the circle indicates the relative frequency of the haplotype.

8   **Table S1. Primer sequences.**

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1   **Tables**

2   **Table 1. Summary statistics by geographic and genetic region.** n = number of individuals;
3   N_H = unique haplotypes; N_P = polymorphic loci; H = haplotype diversity; π = mean number
4   of pairwise differences between sequences; significance is indicated for Tajima's D and Fu's
5   F_S test statistic where * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

Geographic Region	Genetic Region	n	N_H	N_P	H	π	Tajima's D	Fu's F_S
All sequences	HVR + CytB	229	222	205	0.999 ± 0.000	23.12 ± 10.21	-1.03	-23.63**
Ross Sea	HVR + CytB	81	80	124	0.999 ± 0.002	18.49 ± 8.28	-0.903	-22.84***
East Antarctic & Weddell Sea	HVR + CytB	148	145	171	0.999 ± 0.001	22.15 ± 9.81	-0.930	-23.80**
All sequences	HVR	229	220	164	0.999 ± 0.001	20.62 ± 9.14	-0.835	-23.67**
Ross Sea	HVR	81	76	109	0.997 ± 0.003	16.81 ± 7.56	-0.836	-17.21*
East Antarctic & Weddell Sea	HVR	148	144	138	0.999 ± 0.001	19.58 ± 8.71	-0.758	-23.85**
All sequences	CytB	229	59	41	0.864 ± 0.016	2.94 ± 1.54	-1.651*	-21.79***
Ross Sea	CytB	81	26	15	0.876 ± 0.028	1.99 ± 1.14	-0.979	-3.43
East Antarctic & Weddell Sea	CytB	148	41	33	0.797 ± 0.031	2.96 ± 1.56	-1.482*	-12.92***

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1 **Table 2. Pairwise genetic differentiation between colonies.** Pairwise θ_{ST}s are presented
2 below the diagonal, and associated *p*-values above the diagonal. Significance is indicated by
3 bold text, where * denotes p < 0.05, ** denotes p < 0.01, *** denotes p< 0.001.

4

	Gould Bay	Halley Bay	Fold Island	Auster	Amanda Bay	Pointe Géologie	Cape Washington	Cape Crozier
Gould Bay		0.596	0.731	0.560	0.186	0.129	**0.000**	**0.006**
Halley Bay	-0.027		0.566	0.798	0.708	0.301	**0.002**	**0.008**
Fold Island	-0.050	-0.032		0.515	0.323	0.462	**0.000**	**0.007**
Auster	-0.026	-0.086	-0.027		0.595	0.797	**0.000**	**0.000**
Amanda Bay	0.055	-0.058	0.014	-0.038		0.576	**0.000**	**0.000**
Pointe Géologie	0.091	0.029	-0.012	-0.085	-0.033		**0.000**	**0.000**
Cape Washington	**0.355*****	**0.440****	**0.468*****	**0.567*****	**0.617*****	**0.596*****		0.509
Cape Crozier	**0.213****	**0.266****	**0.256****	**0.432*****	**0.447*****	**0.428*****	-0.011	

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1   **Figure Legends**

2   **Figure 1: Sample locations of emperor penguin colonies.** The number of sequences

3   obtained from each location is shown in brackets. Red points indicate the origin of modern

4   samples, the blue point indicates the origin of the subfossil samples.

5   **Figure 2: Extended Bayesian skyline plots showing the change in effective female**

6   **population size (Ne_f).** Solid lines show the median estimate; dotted lines show the 95%

7   highest posterior density interval. a) EAWS colonies; b) Ross Sea colonies; c) the Antarctic

8   temperature anomaly (the difference from the average of the last 1000 years) as estimated

9   from the EPICA Dome C ice core (Jouzel *et al.*, 2007), with the ice core location indicated in

10   red.

11   **Figure 3: Phylogenetic relationships among individuals.** Magenta - EAWS individuals;

12   green - Ross Sea individuals; blue – subfossil individuals. The posterior probabilities are

13   shown for the major, strongly supported clades.

14   **Figure 4. Schematic of contemporary population structure and reconstruction of**

15   **historical conditions.** Sampled colonies are indicated by dots, as in Figure 1. The magenta

16   and green shading indicates population structure as estimated from this study. Lines represent

17   the sea ice edge, as in Gersonde *et al.*, 2005. M-SSI = modern summer sea ice edge; LGM-

18   SSI = LGM summer sea ice edge; M-WSI = modern winter sea ice edge; LGM-WSI = LGM

19   winter sea ice edge, ? = insufficient data to reconstruct the sea ice edge. Penguins represent

20   hypothesised locations of polynya refugia. Emperor penguin picture: © Samuel Blanc /

21   www.sblanc.com.

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