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ORIGINAL PAPER

Release from bats: genetic distance and sensoribehavioural regression in the Pacific field cricket, *Teleogryllus oceanicus*

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Abstract The auditory thresholds of the AN2 interneuron and the behavioural thresholds of the anti-bat flight-steering responses that this cell evokes are less sensitive in female Pacific field crickets that live where bats have never existed (Moorea) compared with individuals subjected to intense levels of bat predation (Australia). In contrast, the sensitivity of the auditory interneuron, ON1 which participates in the processing of both social signals and bat calls, and the thresholds for flight orientation to a model of the calling song of male crickets show few differences between the two populations. Genetic analyses confirm that the two populations are significantly distinct, and we conclude that the absence of bats has caused partial regression in the nervous control of a defensive behaviour in this insect. This study represents the first examination of natural evolutionary regression in the neural basis of a behaviour along a selection gradient within a single species.

Keywords Neuroethology · Genetic isolation · Evolution · Sensory ecology · Island biology

Introduction

Whereas the existence of vestigial characters in organisms has long been documented (Darwin 1859), the mechanisms behind those regressive changes are less well understood (Fong et al. 1995; Borowsky and Wilkens 2002; Romero and Green 2005). Character regression presumably follows the relaxation of selection pressure(s), but it is usually

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difficult to identify what these putative pressures once were. The evasive flight responses of eared insects to the echolocation calls of hunting bats offer a rare opportunity to study a behaviour that is governed by a single sensory modality and evoked by few neurons (Yager 1999). The Pacific field cricket, *Teleogryllus oceanicus* (Orthoptera, Gryllidae) is a flighted insect whose distribution ranges naturally across the South Pacific ocean from Indonesia to French Polynesia (Chopard 1967; Otte and Alexander 1983) and, accidentally to Hawai`i (Kevan 1990). Although the male calling song of *T. oceanicus* is mainly low frequency (LF, 4.5–5.5 kHz (Hill et al. 1972)), this cricket possesses high-frequency (HF, >15 kHz) auditory sensitivity that it presumably uses to detect the echolocation calls of hunting bats while in flight (as observed for *Gryllus bimaculatus* (Popov and Shuvalov 1977)). As well, certain Australian bats, sympatric with *T. oceanicus* have been reported to prey upon gryllids (Vestjens and Hall 1977). Upon hearing pulsed HF stimuli mimicking bat calls, flying female crickets steer their bodies to move away from the source of the sound (negative phonotaxis), in contrast to their positive phonotactic reaction to LF stimuli mimicking the calling songs of male crickets (Moiseff et al. 1978) further suggesting that as in other insects (Miller and Surlykke 2001), one function of HF sensitivity is the detection and avoidance of echolocating bats. *T. oceanicus* uses a portion of its array of auditory receptors tuned to HF plus a pair of interneurons (AN2) to receive and encode the calls of bats and to command its negative phonotactic responses (Pollack 1998). The activity of the AN2 has been shown to be necessary and sufficient for the activation of steering responses away from ultrasound (Nolen and Hoy 1984), and we suggest that this CNS neuron and the behaviour it controls are suitable candidate traits for examining regressive changes arising from differential levels of the selection pressure (i.e. bat predation) that maintain them. In contrast, another interneuron, the ON1 cell participates in both LF and HF circuitry by amplifying side-to-side differences and allowing the insect the ability to localize both conspecific mating song and bat echolocation calls (Faulkes and Pollack 2000). This cell should remain functional in insects that still require it for LF mate-finding purposes even if HF predator detection is no longer a selective force although its HF sensitivity, like that of the AN2 should be regressed if no longer required.

The Pacific is home to bat assemblages whose compositions range from over 75 species (Australia (van Dyck and Strahan 2008)) to areas where they have never existed (French Polynesia (Fullard 1994)). This west to east reduction of a selection pressure on the HF sensitivity of the ears of *T. oceanicus* predicts that as the cricket dispersed from the bat-rich areas of the West Pacific to the bat-poor islands of the East Pacific, its anti-bat behaviour and the neurons that control it have degenerated. Fullard (1994) and Fullard et al. (2004, 2007) observed partial auditory and behavioural regression in endemic versus introduced noctuid moth species from Tahiti but to our knowledge, similar evolutionary changes to the sensoribehavioural circuitry within a single species have not been demonstrated. Zuk et al. (2001) found significant differences in the temporal characteristics of song in Australian versus Oceanic populations of *T. oceanicus*, suggesting that sufficient time and genetic isolation has occurred to result in phenotypic changes to this insect. On the Hawaiian islands of O`ahu and Kaua`i, a large proportion of males of this species have lost the ability to produce long-distance calling songs, presumably in response to the phonotactic behaviour of acoustic parasitoid flies (Zuk et al. 2006; Tinghitella 2008). This shift occurred in fewer than 20 generations, so rapid evolutionary changes are evident in this animal. The purpose of our study was to compare the acoustic sensitivities of the AN2 and ON1 cells and the flight behaviour of female *T. oceanicus* sampled from a bat-rich site (Darwin, Australia) and a bat-free site (Moorea, French Polynesia). We predict that the HF sensitivity of the AN2 and ON1 cells and the anti-bat flight behaviours they govern will be reduced in the bat-released Moorean population. We suggest that to demonstrate evolutionary regression rather than simple phenotypic variability, it is necessary to establish that the two populations under examination are sufficiently separated and not experiencing ongoing gene flow that would dilute the effect of differences in selection pressures. As *T. oceanicus*-colonized islands across the Pacific, it likely experienced founder effects (Mayr 1942) and genetic reorganization by recombination and drift. Colonization is frequently associated not only with the loss of rare alleles and changed allele frequencies, but also with rapid evolutionary differentiation following exposure to novel selection pressures (Reznick and Ghalambor 2001). In opposition to these forces, we expect ongoing gene flow to homogenize populations both genetically and phenotypically (Slatkin 1987). Here, we first estimate neutral genetic differentiation using variation in microsatellite loci amongst seven Pacific populations of this insect to establish that populations at the extreme ends of their distribution are genetically distinct and not experiencing high levels of gene flow and then apply these results to an examination of the neural basis of this insect's anti-bat behaviour.

Materials and methods

Genetic analysis

Samples for DNA analysis were collected from seven locations in Australia and on Pacific Islands (Fig. 1a)

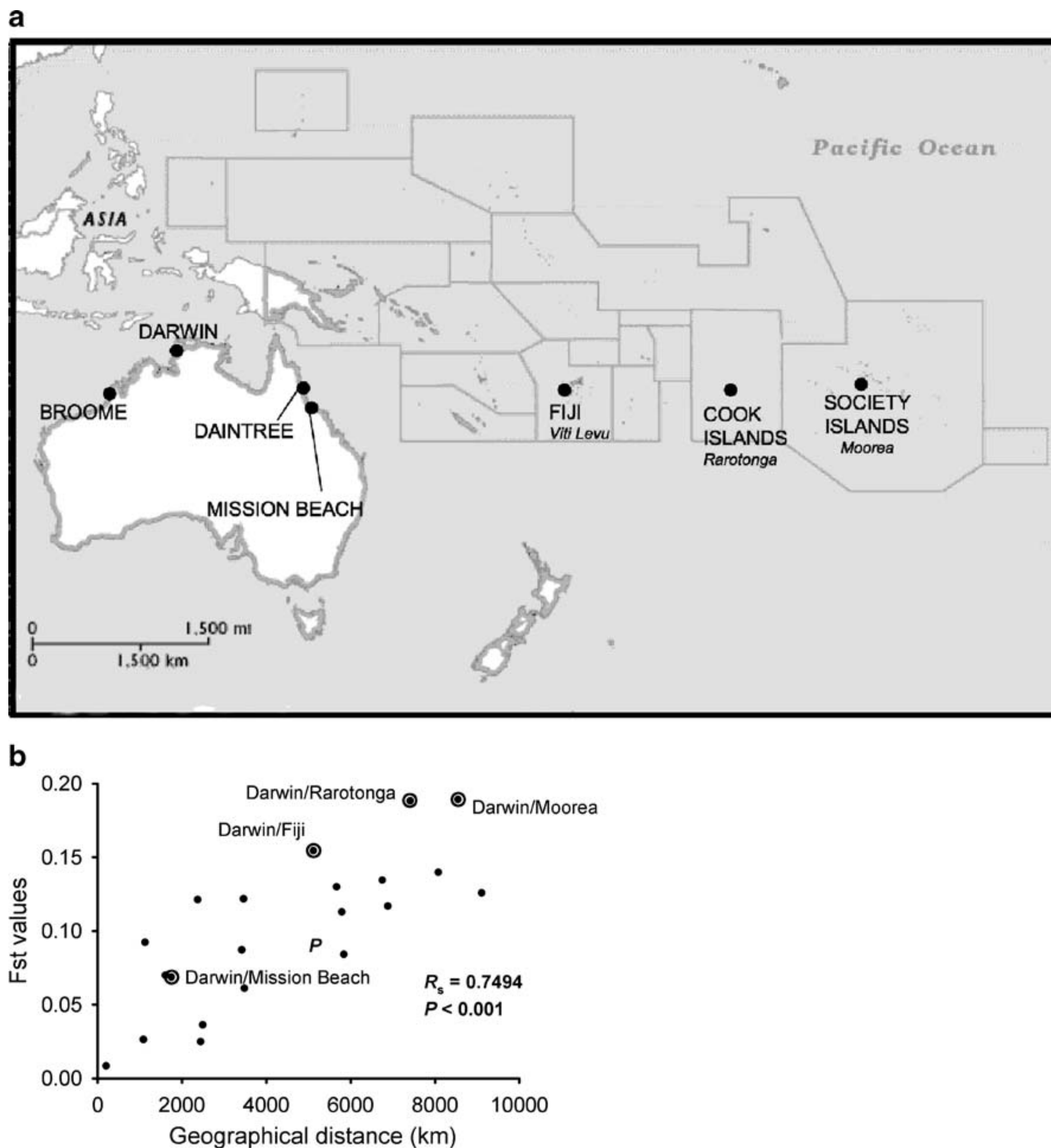

Fig. 1 **a** Geographic locations of crickets sampled for microsatellite analysis. **b** The data from Table 2 are plotted showing that Australian and Pacific Island populations of *T. oceanicus* exhibit a significant correlation ($P<0.05$, Spearman's rank correlation test) between distance and genetic separation suggesting genetic isolation between the two extreme populations (Darwin and Moorea) whose crickets were tested neurally and behaviourally (representative populations outlined in *circles*, correlation performed on all samples)

between 2004 and 2007. One leg was removed from each individual and the hind leg tibial levator and depressor muscles dissected out and stored in ethanol until the time of DNA extraction. DNA was extracted using a standard salt extraction protocol and genetic variation was assayed at seven highly polymorphic microsatellite loci developed specifically for *T. oceanicus* (Beveridge and Simmons 2005; see Electronic supplementary material S1 for description of DNA labelling and PCR amplification methodology).

We screened 137 samples from seven Pacific populations with sample sizes ranging from ten to 25 individuals per population (Table 1). Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were assessed using GENEPOP version 3.4 (Raymond and Rousset 1995). Allelic diversity (for each locus and pooled across the six loci), allelic richness for each locus and population, heterozygosity, and a matrix of pairwise genetic distances (F_{st} estimated by Weir and Cockerham 1984) were also produced using GENEPOP version 3.4 and FSTAT version

2.9.3.2 (for allelic richness; Goudet 1995). Pairwise genetic distances were then used in conjunction with population pairwise geographic distances calculated using Google Earth to test for isolation by distance using the ISOLDE option. A Mantel test performed in ISOLDE assessed how well geographic distance estimated genetic distance in the microsatellite data set. Geographic trends in genetic diversity were assessed by regressing the expected heterozygosities for each population against the distance (kilometres) from Broome, Australia (the furthest west of our sampled populations). We asked whether the seven populations had recently experienced bottlenecks (a severe reduction in effective population size) using BOTTLENECK version 1.2.02 (Piry et al. 1999). BOTTLENECK estimates observed heterozygosity excess as compared with expected equilibrium heterozygosity. We performed the tests using all three options available in BOTTLENECK, the infinite allele model (Kimura and Crow 1964), the stepwise mutation model and the two phase model (probability for SMM 70%, variance 30%) and significance of the results was evaluated with the Wilcoxon signed-rank test ($P<0.05$, Cornuet and Luikart 1996).

Auditory neurophysiology

AN2 cell

We collected wild female crickets and conducted our experiments in the following locations and dates: Moorea, French Polynesia (January–February, 2005; $n=10$) and Darwin, Australia (May, 2005; $n=5$). Crickets were cold-anaesthetized and secured, ventral side up, to a block of modelling clay. Cervical connectives were exposed by removing the prothoracic sternum plus a square of membrane from the neck; the connective ipsilateral to the stimulated ear was then draped over a stainless steel hook electrode referenced to another electrode placed in the abdomen. To reduce background neural activity, the connective was severed anterior to the recording electrode and the contralateral connective was severed at its connections with the prothoracic ganglion. (see Electronic supplementary material S2 for description of electrophysiological methodology).

ON1 cell

ON1 recordings were performed at McGill University using female crickets reared from eggs collected in 2005 from the same sites on Moorea ($n=11$) and Darwin ($n=9$) as where the AN2 recordings were conducted. Crickets were cold-anaesthetized and, after removal of their wings, mid- and hind legs, were attached to a support with warm beeswax-colophonium mixture. The prothoracic ganglion was exposed by ventral dissection, supported on a small metal platform, and bathed in physiological saline (Strausfeld et al. 1983; see Electronic supplementary material S3 for description of electrophysiological methodology).

Flight behaviour

Flight trials were conducted at McGill University using females reared from wild-collected eggs from the Darwin and Moorea populations. Crickets (aged 1–2 weeks after the last moult) were attached to applicator sticks at the pronotum using a beeswax-colophonium mixture, and placed ventral side uppermost in a windstream to induce tethered flight. A fibre-optic light source was positioned so that a shadow of the cricket's abdomen was cast on a photocell array which was partly covered by a V-shaped mask. Movements of the abdomen to the left or right, indicative of attempted left and right turns, respectively (Moiseff et al. 1978), caused the shadow to obscure varying portions of the unmasked portion of the display, yielding a voltage readout that we used to monitor steering attempts (see Pollack and Martins 2007, for example recordings; see Electronic supplementary material S4 for description of flight behaviour methodology).

Results

Genetic analysis

Six of the seven loci we screened were in equilibrium (one population per locus was out after Bonferroni correction), but locus Totri88a was out of equilibrium in >70% of the populations studied. This is consistent with the observation of Beveridge and Simmons (2005) that this locus shows null alleles and appears to be X-linked. We therefore omitted Totri88a for the analyses presented here, but all patterns were consistent with or without it. This left Totri 9a, Totri 54, Totri 55a, Totri 57, Totri 59, and Totri 78. No linkage disequilibrium was observed in these populations, indicating all six remaining loci segregated independently of each other.

We sampled ten to 25 individuals per population and found the total number of alleles pooled across the six loci to vary from 32–78 per population (Table 1). Among the seven populations, the highest allele diversity with an average of 13 alleles per locus was found in Broome and the lowest in Rarotonga and Moorea, both with an average of 5.33 alleles. Allelic diversity (N_a) and allelic richness (AR) decreased roughly from west to east for all six loci, consistent with a spread of the cricket from their native Australia to the islands of the Pacific. Low N_a was noted in Darwin, Australia, relative to other Australian populations

Table 1 Summary table of allelic diversity (per locus and summed across six microsatellite loci), allelic richness (rarefied to a sample size of 4), BOTTLENECK statistics, and the weighted average diversity (H_e) among individuals within populations

Population	N	Allelic diversity (N_a) and richness (AR) by locus						N_a	BOTTLENECK (IAM/SMM/TPM)	H_e by population
		1	2	3	4	5	6			
Broome, Australia	20	10 5.422	11 5.414	19 6.979	15 6.063	12 3.662	11 5.692	78	−/−/−	0.8499
Daintree, Australia	20	10 5.455	11 5.280	13 6.187	10 4.713	8 3.962	9 5.461	61	−/−/−	0.8462
Darwin, Australia	10	6 6.00	5 4.378	7 6.133	9 6.308	1 1.000	7 5.978	35	−/−/−	0.7298
Mission Beach, Australia	23	8 5.260	10 4.560	16 6.840	13 4.296	12 5.171	11 5.662	69	−/−/−	0.8385
Viti Levu, Fiji	20	4 3.251	5 3.499	13 6.362	6 4.194	10 4.411	7 3.898	38	+/−/−	0.7561
Rarotonga, Cook Islands	19	5 3.819	5 3.236	10 6.333	4 3.361	6 4.147	2 1.988	32	+/−/−	0.6859
Moorea, French Polynesia	25	3 2.262	7 4.062	8 5.045	3 2.453	6 3.699	5 2.964	32	+/−/−	0.6398

Allelic richness can be found below allelic diversity for each locus and population. Primers are 1=Totri 9a, 2=Totri 54, 3=Totri 55a, 4=Totri 57, 5=Totri 59, 6=Totri 78 (after Beveridge and Simmons 2005). Bottleneck results were estimated by Wilcoxon signed-rank tests ($P<0.05$), based on the infinite allele model (IAM), stepwise mutation model (SMM), and two-phased model (TPM)

sampled, and this may reflect a population bottleneck or isolation and little gene flow. We found only one allele at Totri 59 in the Darwin population. Fewer samples from Darwin were included in the analysis relative to other populations so, conversely, this may simply reflect sampling bias. However, we looked for heterozygosity excess using BOTTLENECK version 1.2.02, and found that only the island populations (and not Darwin or the other Australian populations) were likely to have experienced recent bottlenecking events. This is reasonable to expect for island populations, but was only noted when considering one of the three mutation models investigated, the infinite allele model (Table 1).

Mean heterozygosity (Supplemental Material S5) in the Australian populations (Broome, Daintree, Darwin, and Mission Beach) was significantly greater than that in the Pacific island populations sampled (Viti Levu, Rarotonga, and Moorea) (H_e=0.8643±0.0209 SE vs. H_e=0.7063±0.0344 SE, P=0.0002, two-tailed t test). The highest genetic diversity was found in the western populations and diversity (H_e) decreased to the east (R^2=0.6606, F=9.7305, P=0.0263). The mean number of alleles across the six microsatellites was also significantly higher in the Australian populations than in the island populations (10.17±0.76 SE vs. 6.06±0.66 SE, P=0.0003 two-tailed). The AR was significantly greater in Australian populations (5.242) than in the island populations (3.832) sampled following permutation tests in FSTAT (P=0.0307). Pairwise genetic variation (F_{st} values) among populations within Australia were low (ranging from a low of 0.0085 to a high of 0.0699), suggesting closer genetic relationships and higher degrees of ongoing gene flow relative to that among Australian and Pacific island populations (where pairwise F_{st} values ranged from 0.0612 to 0.1892). Of the seven populations studied, Moorea and Darwin were the most genetically distinct (Table 2). There was a significant pattern of isolation by distance among the seven sampled populations (illustrated in Fig. 1b (Spearman rank correlation: R_s=0.7494, N (total comparisons)=21, P<0.001)). Figure 1b illustrates that pairwise genetic differentiation among the sampled populations was significantly correlated with distance (Spearman's rank correlation: R_s=0.7494, N (total comparisons) = 21, P<0.001).

Auditory neurophysiology

AN2 cell

Figure 2a shows the median audiograms of the AN2 interneuron of ten specimens of Moorean and five specimens of female Australian *T. oceanicus*. The audiograms are similar in shape showing the typical high-frequency sensitivity of this cell (Moiseff and Hoy 1983; Fullard et al. 2005) but differ significantly (P≤0.05, Mann–Whitney two-tailed U tests) in their thresholds. Except for 2–5 kHz, all of the Moorean individuals are 15–30 dB

Table 2 Pairwise genetic distances (F_{st}, above) and geographic distances (km, below) among the seven populations sampled

Population	Broome	Darwin	Daintree	Mission Beach	Viti Levu	Rarotonga
Darwin	0.0264					
	1,085					
Daintree	0.0249	0.0699				
	2,442	1,607				
Mission Beach	0.0363	0.0687	0.0085			
	2,493	1,749	202			
Viti Levu	0.0841	0.1545	0.0612	0.0871		
	5,836	5,116	3,478	3,418		
Rarotonga	0.1398	0.1883	0.1129	0.1299	0.1213	
	8,080	7,412	5,792	5,666	2,373	
Moorea	0.1258	0.1893	0.1169	0.1345	0.1218	0.0923
	9,107	8,545	6,882	6,752	3,460	1,126

Values were used to test for a pattern of isolation by distance in a Mantel test using the ISOLDE option in GENEPOP (Raymond and Rousset 1995) and were significant at $P<0.003$. See Electronic supplementary material S5 for table of Gene diversities (heterozygosities) among individuals within populations per locus

significantly less sensitive than their Australian counterparts for the entire range of frequencies tested which covers almost the entire frequency range of the most speciose bat assemblage (Australia) (Churchill 1998).

ON1 cell

Figure 2a shows the median audiogram of the ON1 interneuron of 11 specimens of Moorean and ten specimens of Australian *T. oceanicus*. Unlike the AN2 audiograms, the two populations of crickets reveal significant ($P>0.05$, Mann–Whitney two-tailed U tests) ON1 threshold differences at only one stimulus frequency (15 kHz).

Flight behaviour

Figure 2b shows the stimulus thresholds for flight alteration in ten female Moorean and 10 Darwin *T. oceanicus* and indicates that there was no significant ($P>0.05$, Mann–Whitney U test) difference in the stimulus intensities required to elicit a turning towards response when stimulated with the acoustic frequency of the cricket's male calling call (4.5 kHz). Even though the difference of the median thresholds between these two populations was insignificant due to the high variability in the Darwin crickets, the threshold of these insects was over 10 dB lower than that of the Moorea crickets. Moorean crickets however, required a significantly higher intensity of sound to evoke a turning-away response when stimulated by a frequency typically found in bat echolocation calls (30 kHz). Unexpectedly, the neurally determined AN2 thresholds were higher than those measured behaviourally, and we believe that this was due to the different experimental conditions of the animals (dissected for neurophysiology, intact for behaviour) and/or that the neural stimuli were 20 ms compared with the 30 ms used in the behavioural trials.

Discussion

Our genetic analysis of the Pacific populations of *T. oceanicus* indicates that populations that are more distant from one another geographically are also more genetically distinct with the result that Moorean crickets significantly differ from Darwin crickets in neutral genetic differentiation (pairwise F_{st}=0.1893). In fact, of the seven populations we sampled, these two were the most genetically distinct. The reduction in genetic diversity from west to east (as evidenced by significantly lower heterozygosities, allelic diversity, and allelic richness in island relative to Australian populations) and higher neutral genetic differentiation suggests that the Moorean population has evolved in relative genetic isolation for some time. It is difficult to say how much isolation is enough to allow for the appearance of phenotypic characters suited to their bat-free environment, but certainly, some level of isolation is necessary in this regard. In the absence of such isolation, we would expect the counteracting effects of gene flow to homogenize Australian and island populations both phenotypically and genetically (as seems to be the case among Australian populations investigated here, despite the vast inter-population geographic distances within Australia). The additional observation that Moorea has experienced a recent bottleneck suggests again that the island is isolated from Australian populations and that gene flow has not homogenized populations across the cricket's range.

Neural regression

In female *T. oceanicus*, the LF circuit is responsible for the turning-towards flight response in crickets searching for singing, conspecific males, and the HF circuit initiates the turning-away response so as to avoid the attention of echolocating bats. Our results indicate that the ON1 cell, which participates in both circuits (Marsat and Pollack

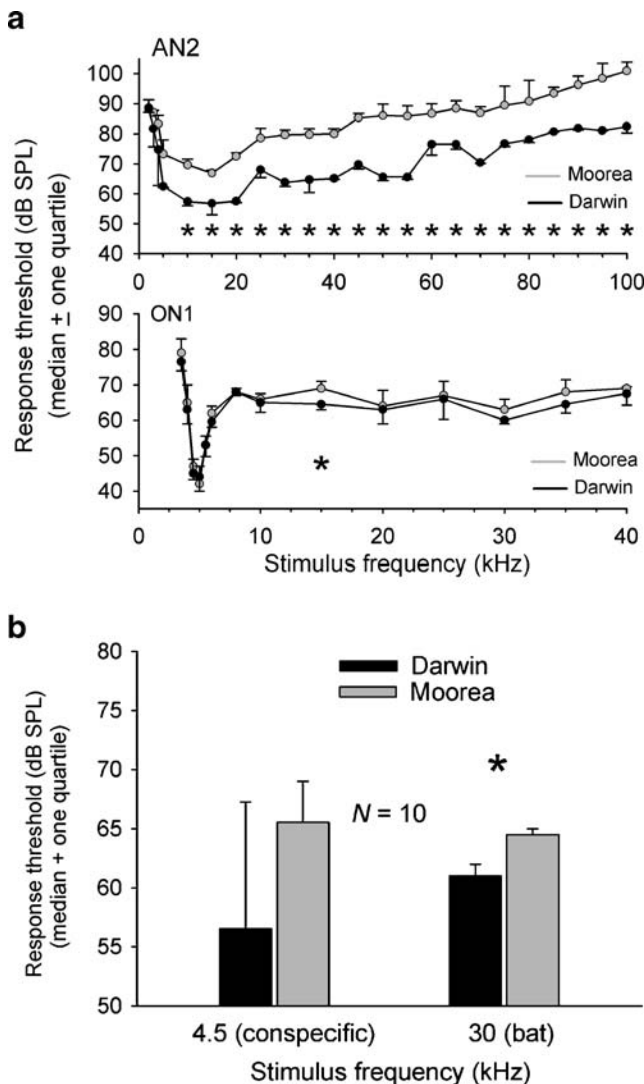

Fig. 2 **a** Median auditory threshold curves of Darwin versus Moorea *T. oceanicus* AN2 and ON1 cells. **b** Stimulus intensities required to elicit turning-towards flight responses to conspecific calling song frequencies and turning-away flight to bat-like echolocation call frequencies in the two populations of crickets. *Asterisks* in both graphs indicate significant differences ($P<0.05$, Mann–Whitney *U* tests)

2007) has remained largely unchanged in crickets from the bat-free habitat of Moorea, but the AN2 cell, which is limited to the HF circuit, has undergone evolutionary regression. The degeneration of the Moorean AN2 cell is only apparent at frequencies of 10 kHz and higher and resembles that observed in other bat-released insects: Tahitian moths (Fullard et al. 2004), flightless mantises (Yager 1990) and crickets (Pollack and Martins 2007), and diurnal moths (Fullard et al. 1997; Surlykke et al. 1998). The absence of total regression of the AN2 cell in bat-released crickets may be due to its possible role in detecting the HF components of male aggressive and courtship songs (Hutchings and Lewis 1984; Harrison et al. 1988). In the absence of bats, this cell could continue to function as a close-range HF detector and allow for a reduction in its sensitivity since the song intensities of close conspecifics (>90 dB (Balakrishnan and Pollack 1996)) would be greater than those of the echolocation calls of distant, searching bats.

It is unclear why a similar loss of HF sensitivity did not occur in the ON1 interneuron since this cell is involved with enabling the cricket to localize bat-like sounds via its effects on the AN2 cell (Marsat and Pollack 2007). A study on a wing-dimorphic species, *Gryllus texensis*, showed that in short-winged, flight-incapable individuals (i.e. not exposed to predation by aerially hawking bats), thresholds of both AN2 and ON1 to high frequencies were higher than in long-winged, flight-capable individuals, whereas sensitivity to low frequencies was not significantly different between the two morphs (Pollack and Martins 2007). However, when long-winged crickets lost the ability to fly through histolysis of flight muscles, there was a corresponding loss of high-frequency sensitivity in AN2, but not in ON1. Thus, developmental change in high-frequency sensitivity appears to be regulated, perhaps hormonally (Narbonne and Pollack 2008), independently in the two interneurons, as indeed has occurred in the present case. Presumably, regressive changes to both cells would appear more slowly on an evolutionary scale and, as with the persistence of the bat-released AN2 cell, ON1 may continue to process the HF harmonics of male song which are necessary to evoke phonotaxis in females (Latimer and Lewis 1986).

Behavioural regression

Nolen and Hoy's (1984) discovery of the command status for the AN2 cell in *T. oceanicus* predicts that if its responsiveness diminishes so will the flight response that it controls, and our results support this. We conclude that Moorean crickets, released from the need to detect the HF calls of bats, have increased thresholds in their avoidance flight response as a result of the regressed condition of their AN2 cells. Although the LF behavioural responses are statistically similar, we recognize that a trend exists for Moorean crickets to be less sensitive to calling song frequencies perhaps reflecting a reduction in flight activity or ability in these island insects. We note that at the frequency used in the flight response trials (30 kHz), there is a considerable dissimilarity in the median differences of thresholds between the two cricket populations measured neurally at the AN2 level (15.8 dB) versus those measured behaviourally (3.5 dB). We suggest that this discrepancy relates to differences in the threshold for single spikes at threshold compared with the high spike rates required to elicit a behavioural action and may describe a neural safety

margin that exists in this insect to avoid responding to innocuous, background sounds. Indeed, AN2 evokes behavioural responses only when it fires at high rates (Nolen and Hoy 1984; Marsat and Pollack 2006). The fact that crickets still exhibit avoidance flight, albeit at higher sound intensities, parallels the results of Fullard et al. (2004) who observed partial behavioural regression in endemic Tahitian noctuid moths and suggests that not enough time has passed in genetic isolation for this character to have completely disappeared. The gradual vestigialization of anti-bat flight in *T. oceanicus* is in stark contrast to the rapid and complete loss of singing in Kauaian crickets caused by the active selection pressure of parasitoids (Zuk et al. 2006) and supports the theory of genetic drift as the causative agent underlying passive character regression (Haldane 1933).

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The forward primers were labelled with either 6-FAM, HEX (Integrated DNA Technologies), or PET (Applied Biosystems). Each 15 µL PCR reaction contained 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1.5 mM or 3.5 mM $MgCl_2$ (see Beveridge and Simmons 2005 for details; Invitrogen), 200 µM of each dNTP (Invitrogen), 250 nM of the forward primer (labelled and unlabelled in a ratio of 1:5), 250 nM of the reverse primer (Integrated DNA Technologies), 1 unit of Platinum Taq DNA polymerase (Invitrogen) and ~10ng of DNA. PCR amplification was performed with the following cycling conditions: 94°C for 1 min, then 30 cycles at 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, and finally 72°C for 45 minutes. We analyzed the products on an ABI 3100 Genetic Analyzer and sized using a LIZ internal size standard and GeneMapper V3.7(2) software.

Neural activity was amplified (Grass P15 AC amplifier) and displayed online and stored in a PC laptop using an oscilloscope-emulating sampling board (50 MHz, ADC 212/3; Pico Technology) and analysis program (PicoScope 5.10.7; Pico Technology). The ears were exposed to pulsed synthetic sounds (20 ms, 500 µs rise/fall time, 2 s^{-1}) generated by a customised MATLAB application and delivered to the preparation via a high-speed data acquisition PCMCIA card (National Instruments, BNC 2110), amplifier (Avisoft Bioacoustics, model 70101) and speaker (Technics leaf tweeter, EAS 10TH400B, speaker response (5 – 100 kHz): harmonic distortion at 90 dB SPL, < -40 dB) positioned 30 cm from the cricket's ear. The auditory preparation and speaker were mounted in a sound absorbing Faraday cage. Intensities were recorded as millivolts peak-to-peak delivered to the speaker and converted to peak equivalent sound pressure levels (dB peSPL) (rms re 20 µPa) from equal-amplitude continual tones as measured with a Brüel and Kjær (B&K) Type 4135 6.35-mm microphone and Type 2610 B & K measuring amplifier. The entire system was calibrated before and after the study with a B & K Type 4228 pistonphone. We generated audiograms (threshold–response curves) by broadcasting pulses in a randomized order of frequency presentation (2-5 kHz in 1 kHz intervals, 10-100 kHz in 5 kHz intervals) and increasing the pulse amplitude until they just elicited two AN2 action potentials three times in a row from the cervical connective. Background spiking activity in this cell is usually very low (< 1 spike/second) facilitating the on-line recognition of neural response.

The identified auditory interneuron, ON1, was recorded extracellularly from its soma-contralateral processes using a blunt microelecrode (5-10 MOhm) filled with 1M NaCl. Responses of ON1 are unambiguously identifiable by its lower threshold to electrode-contralateral (i.e. soma-ipsilateral) stimuli, and its lower threshold, yet longer latency, to 4.5 kHz than to 30 kHz stimuli (Faulkes and Pollack 2000). Pure-tone sound stimuli (3 kHz – 40 kHz; 30 ms duration including 5 ms rise and fall ramps, presented at a rate of 0.5 Hz) were generated by a National Instruments A/D card (PCI 6251; sampling rate 100 kHz) and broadcast from Radio-Shack 40-1310 loudspeakers. Customized Matlab software controlled a programmable attenuator such that the frequency response of the system was flat (+/- 1dB). Threshold at each frequency was defined as the minimum sound level that elicited at least two spikes on three of five repetitions, and was measured online (resolution; 1 dB) in an automated manner by customized software (Matlab).

Sound stimuli were either 30 ms pulses (including 5 ms rise and fall ramps) with carrier frequency of 30 kHz, or 6 sec-long trains of 30 ms pulses, with carrier frequency of 4.5 kHz and pulse period of 62.5 ms. Earlier work (e.g. Pollack and El-Feghaly 1993) showed that these two stimulus types are, respectively, aversive and attractive for *T. oceanicus* females. Stimuli were presented in blocks of 5 trials, with 10 sec pauses between trials and at least one minute between blocks. Stimulus type (aversive or attractive) and sound level was constant within each block. Threshold was defined as the lowest sound level that evoked a response on at least three of the five trials of a block. We began with a stimulus level that, based on earlier studies, we expected to be above threshold (60 dB SPL for the attractive stimulus; 80 dB for the aversive stimulus; if subthreshold, we increased sound level in 10 dB steps until threshold was attained). We then decreased sound level in steps of 10 dB until the stimulus was below threshold, and then increased in steps of 2 dB until again reaching threshold. For each trial, we determined whether or not a response occurred by comparing post-stimulus abdominal position with mean abdominal position during the 1-second period immediately preceding stimulus onset. A response was considered to have occurred if the post-stimulus position departed from the range defined by the pre-stimulus mean ± 4 s.d. within the period 10 ms – 200 ms after stimulus onset for the aversive stimulus, and 10 ms – 5 sec for the attractive stimulus.

Behavioural and electrophysiological experiments were performed in chambers lined with acoustic foam to reduce echoes. Sound stimuli were produced and responses recorded by National Instruments A/D - D/A boards (sampling rates: 100 kHz for sound stimuli; 10 kHz for electrophysiological recordings; 1 kHz for behavioural recordings.

Gene diversities (heterozygosities) among individuals within populations per locus.

	Totri 9a	**Totri 54**	**Totri 55**	**Totri 57**	**Totri 59**	**Totri 78**
Broome	0.8807	0.8743	0.9645	0.9123	0.5790	0.8960
Darwin	0.9583	0.7750	0.9750	0.9643	0*	0.9500
Daintree	0.8922	0.8758	0.9271	0.7786	0.7178	0.8934
Mission Beach	0.8792	0.7900	0.9643	0.6961	0.8387	0.8964
Viti Levu	0.6357	0.6855	0.9327	0.8042	0.7909	0.7295
Rarotonga	0.7778	0.6025	0.9375	0.6964	0.7885	0.4875
Moorea	0.4316	0.7658	0.8561	0.4629	0.6957	0.6331

* In the 10 individuals tested in the Darwin population, we found only one allele at locus Totri59.

Sound levels were calibrated using B&K instruments (Type 4135 microphone, Type 2610 SPL meter).