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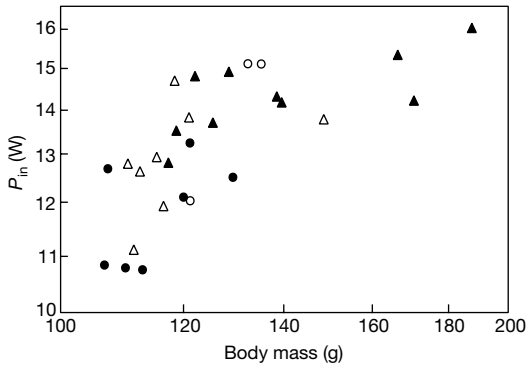

Figure 1 Total metabolic power input (P_{in}) in relation to average body mass (m_b) in red knots flying at 15 m s^{-1}. Different symbols denote different individuals. An analysis of covariance (ANCOVA) with individual as factor and $\log_{10} m_b$ as covariate revealed significant effects of $\log_{10} m_b$ ($F_{1,23} = 7.00$, $P = 0.01$) and individual ($F_{3,23} = 3.11$, $P = 0.046$) on $\log_{10} P_{in}$. The interaction term was not significant (that is, the slope relating $\log_{10} P_{in}$ to $\log_{10} m_b$ did not differ between individuals) and was excluded from the model. The common slope relating $\log_{10} P_{in}$ to $\log_{10} m_b$, correcting for individual differences in intercept, was 0.35 (95% CI 0.08–0.62). Individual intercepts were 0.41, 0.39, 0.41 and 0.35.

Carrying large fuel loads during sustained bird flight is cheaper than expected

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Birds on migration alternate between consuming fuel stores during flights and accumulating fuel stores during stopovers. The optimal timing and length of flights and stopovers for successful migration depend heavily on the extra metabolic power input (fuel use) required to carry the fuel stores during flight[1,2]. The effect of large fuel loads on metabolic power input has never been empirically determined. We measured the total metabolic power input of a long-distance migrant, the red knot (*Calidris canutus*), flying for 6 to 10 h in a wind tunnel, using the doubly labelled water technique[3]. Here we show that total metabolic power input increased with fuel load, but proportionally less than the predicted mechanical power output from the flight muscles. The most likely explanation is that the efficiency with which metabolic power input is converted into mechanical output by the flight muscles increases with fuel load. This will influence current models of bird flight and bird migration. It may also help to explain why some shorebirds, despite the high metabolic power input required to fly, routinely make nonstop flights of 4,000 km longer[4].

Aerodynamic theory predicts that the mechanical power output the flight muscles must generate, to support the weight of the bird and to overcome the drag of the body and wings, increases with fuel load[5–7]. The available evidence indicates that the aerodynamic models correctly describe the essential physical processes involved[8]. The proposed parameter values, and hence the generated predictions, are also reasonably realistic[8]. However, mechanical power output accounts for only part of the total metabolic power input of a flying bird[5–8]. When the flight muscles contract, most of the supplied fuel energy is lost as heat. Heat is also lost when respiration and circulation deliver fuel and oxygen to the flight muscles[5,6] and in metabolic functions not directly involved in the generation of aerodynamic work. In current models of bird flight, metabolic power input is usually calculated from mechanical power output predicted from aerodynamic theory[5,6,8]. The flight muscles are assumed to convert supplied fuel energy into work with a constant efficiency, the basal metabolic rate is added on top, and a multiplication factor is applied to account for increased respiration and circulation[5,6,8]. The greatest limitation to these models is the uncertainty of the assumed muscle efficiency[8].

We measured metabolic power input, using doubly labelled water, in four red knots flying at different body masses at 15 m s^{-1}, in a wind tunnel[9]. The average total metabolic power input during 28 flights lasting between 6 and 10 h was 13.5 W at an average mass of 128 g. The metabolic power input increased allometrically with body mass within individuals with an exponent of 0.35 (95% confidence interval (CI) 0.08–0.62, Fig. 1). Flight muscle efficiency, estimated from metabolic power input and predicted mechanical power output, increased with fuel load (Fig. 2). The estimated level of flight muscle efficiency is uncertain because of uncertainties in the assumptions that come into the calculation of mechanical power[8,10,11]. However, the significant change in efficiency with fuel load is robust to these uncertainties in the assumptions.

Birds need to carry large fuel stores to perform long migratory flights. The weight of these fuel stores increase the mechanical power output required to fly. Power output from the flight muscles can be controlled by altering the strain rate (wingbeat frequency or amplitude) or the proportion of muscle fibres used[8,12]. Both of these alternatives affect muscle efficiency[8,12]. If the flight muscles are optimized to give maximum efficiency at one mechanical power output, efficiency will be reduced as power output changes away from this optimum. Adjustment of flight muscle size and the use of intermittent flight have been suggested to be adaptations for maintaining maximum muscle efficiency as body mass varies owing to build-up and use of fuel stores during migration[6,12]. Red knots do not use intermittent flight, but they do adjust flight muscle size to their current weight (measured by ultrasound in a parallel study of the same birds)[13]. Great knots (*Calidris tenuirostris*), near relatives of the red knots, reduce flight muscle size during long migratory flights[14]. The larger flight muscles at high weights do not seem sufficiently powerful to maintain manoeuvrability, as caged knots are considerably easier to catch when heavy than when light. Several studies have documented how take-off ability in birds is

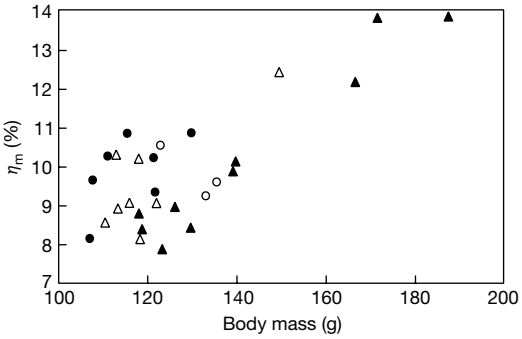

Figure 2 Flight muscle efficiency (η_m) in relation to body mass (m_b). η_m is calculated from measured metabolic power input and mechanical power output predicted from aerodynamic theory (see Methods). An ANCOVA with individual as factor and m_b as covariate showed significant effects of m_b ($F_{1,23} = 71.9$, $P < 0.001$), and individual ($F_{3,23} = 5.86$, $P = 0.004$) on η_m. The interaction term was not significant and was excluded from the model: $\eta_m = \sin(a + 0.0014 m_b)^2$, where $a = 0.14$, 0.15, 0.13 and 0.16 for the individual birds. Changes in the assumed induced drag factor, body drag coefficient and profile power ratio within realistic values changes the predicted level of η_m but does not remove the significant effect of m_b on η_m. The η_m values are within the range of other recent studies[8].

hampered at high body weights[15,16]. This decrease in manoeuvrability, together with the observed increase in muscle efficiency with body mass, suggests that there may be a trade-off between these two aspects of flight performance. Maintaining flight muscles at a size that gives maximum muscle efficiency in cruising flight may be incompatible with maintaining a large muscle spare capacity for transient manoeuvres, such as sprints, steep climbs and rapid turns. The advantage of maintaining a sizeable muscle spare capacity could be more important when birds are resident in the area and flights are mostly short with a large proportion of transient manoeuvres, for example at stopover sites, winter quarters and breeding quarters. During long migratory flights with heavy fuel loads, selection may instead favour maximal muscle efficiency at the expense of a reduced manoeuvrability. The change in muscle efficiency with body mass may be a result of the birds regulating flight muscle size to maintain an optimal balance between different aspects of flight performance (of which muscle efficiency is one) under changing ecological circumstances. Birds on migratory flights are expected to select an optimal flight speed that changes with body mass[17]. During our experiments, birds always flew at one fixed speed. If the flight muscles are adapted to operate efficiently when cruising at an optimal flight speed, part of the variation in muscle efficiency that we observe may be explained by flight speed deviating from the optimal flight speed as body mass changes. An increase in efficiency with body mass implies that the value of a unit of fuel, in potential flight distance, decreases less with fuel load than is assumed in current models of bird migration and flight. The penalty on flying with heavy fuel loads will thus be considerably smaller than previously thought. This significantly alters predictions about the optimal way in which birds organize their migratory journeys and may explain why many shorebirds regularly carry heavy fuel loads and divide their migratory journeys into only a few long nonstop flights measuring 4,000 km or more[4]. □

Methods

Animals and wind tunnel

Four red knots (*Calidris canutus* L.) caught as adults in the Dutch Wadden Sea in 1998 were trained to fly in a wind tunnel[9]. Holding conditions are described in ref. 13. Birds were freely fed trout food pellets supplemented by mealworms (*Tenebrio* sp.) and variation in body mass was due to natural endogenously controlled seasonal changes[18]. Safety constructions in the test section included a net upstream, a nylon-covered polyurethane foam ceiling and a net covering the top of the open section. All birds had intact flight feathers and flew in a natural manner, well clear of test section walls during experiments (Sept.–Dec. 1998, June 1999, Sept.–Dec. 1999 and May 2000). Equivalent air speed was 15 m s^{-1} (within the range for free-flying red knots on migration[19,20]), turbulence 1% of air speed[9], air density 1.22–1.30 (average: 1.25) kg m^{-3} and temperature 4.7–14.5 (average: 10.2) °C.

Experiments

At 07:30 local time, after being without food for at least 12 h, the focal bird was injected quantitatively (±0.001 g, Mettler PM480 DeltaRange), subcutaneously, above the pectoral muscles, with 0.4 ml (10 h flights) or 0.3 ml (6, 7 and 8 h flights) of doubly labelled water (DLW) mixture. Background blood samples were collected before injection. The bird was kept for one hour in a dark chamber (0.3 m × 0.4 m × 0.3 m) for equilibration of injected isotopes with body water. After this, it was weighed (Mettler PM480 DeltaRange) and a blood sample taken to determine initial enrichment. Flights, lasting 10 h ($n = 17$), 8 h ($n = 1$), 7 h ($n = 1$) or 6 h ($n = 9$), started after a 15-min rest. After the flight the bird was weighed, a blood sample was taken to determine final enrichment and the bird was reinjected with 0.3 ml DLW mixture. After one hour in the small dark chamber, the bird was again weighed and a last blood sample taken to investigate changes in the body water pool during flight on the basis of the principle of isotope dilution[21]. All blood samples were taken by puncturing the brachial vein and collecting 15 μl of blood in each of 5 to 8 microcapillary tubes. The tubes were immediately sealed in a flame and stored at 4 °C until analysis. During the first 20 flights birds were weighed after 1, 2, 4, 6 and 8 h, a procedure lasting less than 2 min. On four occasions flights were interrupted for 4 to 21 min because the bird decided to land. Air temperature, air density, interruptions, season or duration of flight did not have a significant effect on the DLW measurements. On average 96% of the time and 99.6% of the energy covered by the DLW measurements was spent in flight. Average mass decrease during experimental flights was 1.09 (standard deviation, s.d. = 0.166) g h^{-1}.

Isotope analysis

Isotope enrichments of blood samples and DLW mixtures for injections were determined in quadruplicate at the Centre for Isotope Research, University of Groningen, The Netherlands[22]. For every four blood samples, one internal enriched water standard was analysed in quadruplicate to monitor the stability of the infrared mass spectrometer and six gas standard samples for each isotope were used to monitor cross-contamination.

Fractional turnover for ^{2}H and ^{18}O and CO_2 production were calculated according to equations (3) and (5) in ref. 22, using the first background isotope enrichment for each bird in each season. Dilution spaces were determined separately for ^{2}H and ^{18}O, before and after the experimental flights, using the plateau method[3,22,23]. The proportion of water efflux through evaporative pathways is assumed to be 0.85 as determined for a red knot flown under similar circumstances (A.K., personal observations). The calculated CO_2 production is not sensitive to the assumed proportion. A proportion of 0.5 will increase the estimated CO_2 production by 0.8%. Error analyses have revealed that the sensitivity of the DLW method to analytical errors is strongly related to the difference between ^{18}O and ^{2}H turnover rates[3]. In birds, the fractional ^{18}O turnover rate is typically about 1.2–1.6 times the ^{2}H turnover rate[3]. However, for the red knots flying in the wind tunnel this value was on average 3.18 (s.d. = 0.269, $n = 28$). This phenomenon, in combination with the long flight duration, and quadruplicate isotope analyses, will have resulted in much better precision levels for the experimental birds than usually reported in avian studies. Total metabolic power input (P_{in}) is calculated using an energy equivalent of CO_2 of 14.1 (kJ g^{-1})[24] and correcting for energy expended during time not spent flying assuming a resting power of 1.5 times the basal metabolic rate (BMR). Basal metabolic rate was derived from a study of red knots under similar holding conditions[25].

Muscle efficiency and the value of fuel

Mechanical power output (P_{out}) is calculated[6] using the average body mass (m_b, in g) and air density for each flight and the wingspan and aspect ratio (a.r. in m^2) for each bird[13], assuming an induced drag factor of 1.2 (ref. 6), a body drag coefficient of 0.1 (ref. 10) and a profile power ratio of (8.4/a.r.)[11]. Body frontal area (S_b, in m^2) was taken as $0.00668\, m_b^{0.606}$ (average for 22 red knots; B. Spaans, personal communication). Muscle efficiency is calculated as $P_{out}/(P_{in}/1.1 - \mathrm{BMR})$[6] and was arcsine transformed before statistical tests.

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Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3

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Toll-like receptors (TLRs) are a family of innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens, and induce antimicrobial immune responses[1,2]. Double-stranded RNA (dsRNA) is a molecular pattern associated with viral infection, because it is produced by most viruses at some point during their replication[3]. Here we show that mammalian TLR3 recognizes dsRNA, and that activation of the receptor induces the activation of NF-κB and the production of type I interferons (IFNs). TLR3-deficient ($TLR3^{-/-}$) mice showed reduced responses to polyinosine–polycytidylic acid (poly(I:C)), resistance to the lethal effect of poly(I:C) when sensitized with D-galactosamine (D-GalN), and reduced production of inflammatory cytokines. MyD88 is an adaptor protein that is shared by all the known TLRs[1]. When activated by poly(I:C), TLR3 induces cytokine production through a signalling pathway dependent on MyD88. Moreover, poly(I:C) can induce activation of NF-κB and mitogen-activated protein (MAP) kinases independently of MyD88, and cause dendritic cells to mature.

Viral infection of mammalian cells results in activation of an innate immune response mediated by type I IFNs, IFN-α and IFN-β, and other cytokines, including interleukin (IL)-6 and IL-12 (refs 4, 5). While IFNs inhibit virus replication, IL-6 and IL-12, which are also induced by bacterial infections, elicit cytotoxic responses needed for elimination of intracellular pathogens. Mammalian TLRs recognize lipopolysaccharide (LPS) and other microbial products[1,6–10]. Whereas the receptors for LPS are expressed on the cell surface, dsRNA is known to bind only intracellular targets, including the dsRNA-dependent protein kinase (PKR)[11]. However, cells derived from PKR-deficient ($PKR^{-/-}$) mice still respond to poly(I:C), a synthetic dsRNA analogue, suggesting the existence of another receptor, which recognizes dsRNA[12,13].

To test whether dsRNA can be recognized by a TLR, 293T cells

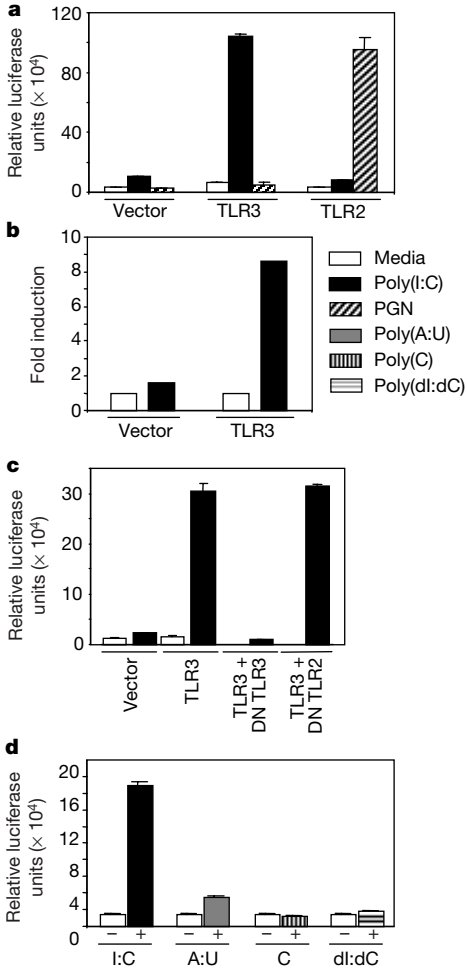

Figure 1 TLR3 specifically signals for NF-κB activation in response to poly(I:C). **a**, 293T cells were transiently transfected with 50 ng of human TLR3, TLR2 or empty pcDNA3 vector together with an NF-κB luciferase reporter. Luciferase activity in cells treated with 25 μg ml^{-1} poly(I:C) or 10 μg ml^{-1} PGN or untreated (media) cells was measured. **b**, Luciferase activity in CaCo-2 cells transiently transfected with 500 ng of empty vector or TLR3 DNA, together with 200 ng NF-κB luciferase reporter and stimulated with 25 μg ml^{-1} poly(I:C). **c**, 293T cells transiently transfected with expression vector for TLR3 or empty vector, together with NF-κB luciferase reporter and, where indicated, 1 μg of dominant negative (DN) TLR3 or DN TLR2 DNAs. NF-κB-induced luciferase activity in cells treated with 25 μg ml^{-1} poly(I:C) or untreated cells was measured. **d**, Transfection of RAW 264.7 macrophages with a NF-κB luciferase reporter. Luciferase activity in cells treated with 20 μg ml^{-1} poly(I:C), poly(A:U), poly(C) or poly(dI:dC), or untreated cells.