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LETTER

Variable and complex food web structures revealed by exploring missing trophic links between birds and biofilm

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Abstract

Food webs are comprised of a network of trophic interactions and are essential to elucidating ecosystem processes and functions. However, the presence of unknown, but critical networks hampers understanding of complex and dynamic food webs in nature. Here, we empirically demonstrate a missing link, both critical and variable, by revealing that direct predator-prey relationships between shorebirds and biofilm are widespread and mediated by multiple ecological and evolutionary determinants. Food source mixing models and energy budget estimates indicate that the strength of the missing linkage is dependent on predator traits (body mass and foraging action rate) and the environment that determines food density. Morphological analyses, showing that smaller bodied species possess more developed feeding apparatus to consume biofilm, suggest that the linkage is also phylogenetically dependent and affords a compelling re-interpretation of niche differentiation. We contend that exploring missing links is a necessity for revealing true network structure and dynamics.

Keywords

Behavioural ecology, feeding ecology, foraging behaviour, functional morphology, omnivory, phylogeny, tongue spine, trophic relationship, wader.

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INTRODUCTION

Food webs are a network representing trophic interactions in ecosystems. Given the important effects of food web structure on population to ecosystem dynamics (Bascompte 2010), identifying full web structure and assessing the ecological implications are fundamental to understand ecosystem processes and functions. Comprehension of food web structure is necessarily based on the structure of networks (links and nodes) being fully 'known'. However, such prerequisites are often not assured. Understanding of ecological networks remains incomplete; fundamental problems in veracity could arise if unknown, but critical networks are present in the real world (Clauset *et al.* 2008).

A major impediment in determining food web structure stems from the difficulty in identifying interspecific links. In general, the discovery of new interactions in networks derives from extensive empirical studies (Bascompte 2010). Furthermore, the presence or absence of a trophic link is modulated by diverse determinants, including species morphological and behavioural traits, phylogenetic constraints and the environment (Kondoh 2003; Cattin *et al.* 2004; Petchey *et al.* 2008; Carnicer *et al.* 2009; Ings *et al.* 2009; Valdovinos *et al.* 2010). These diverse determinants and their properties vary non-linearly, and, in consequence, the strength of the linkages varies in different spatial and temporal (even evolutionary) scales.

Here, we empirically show that a missing and critical trophic link does exist by exposing extensive prey-predator relationships between shorebirds (waders) and biofilm. Subsequently, we show that the strength of this missing link is differentially mediated by node properties (predator species traits), the environment that determines node properties (food density) and evolutionary history (phylogenetic constraints), and propose ecological and evolutionary implications of biofilm feeding.

Although shorebirds prey on invertebrates, such prey cannot account for their complete diet (Zwarts *et al.* 1990; Colwell 2010). A recent study demonstrated that two sandpiper species consume surficial intertidal biofilm (Kuwae *et al.* 2008; Mathot *et al.* 2010), a hitherto unsuspected food comprised of microbes, their extracellular mucus substances and detritus (Characklis & Marshall 1989). However, the extent and determinants of the biofilm feeding phenomenon among shorebirds and the spatial and temporal scales of such behaviour remain unknown. Here, we combined empirical evidence from a synthesis of ecological (stable isotopes), physiological (energy budgets), behavioural (foraging

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videos), morphological (feeding apparatus) and phylogenetic analyses to demonstrate that biofilm feeding is indeed widespread.

MATERIALS AND METHODS

Study site

We investigated the diet of three sandpiper species (Red-necked Stint *Calidris ruficollis*, Western Sandpiper *C. mauri* and Dunlin *C. alpina*) using video image analyses of foraging behaviour, carbon and nitrogen stable isotope analyses, and energy budget analyses at different migratory stages on six intertidal mud- and sand-flat sites along the Pacific coasts of Japan and Canada (see Table S1 in Supporting Information).

Visually fresh sandpiper droppings were collected at the sites. To examine how much biofilm formed on sediment surfaces contributes to the food source for the sandpipers, surface sediments (up to c. 1 mm depth) were collected from the same sites as the bird droppings. We used a toothbrush to collect the thin layer of surface sediment, commensurate with the micro-topography of the surface as well as the action of shorebird tongue spines. No microinvertebrates were visible to the naked eye in the sampled sediments. Sedimentary organic matter (c. 0–2 cm depth) was collected from the sites as a mixture of visible food sources, using a 1-mm mesh sieve. Microscopy (20×) revealed that the samples contained microinvertebrates (e.g. *Capitella* polychaetes), dwelling tubes of macroinvertebrates and detritus. Also, macroinvertebrates were collected by digging at the sites. All samples were dried and powdered for analyses of stable isotope ratios, total organic carbon content and energy content (see below).

Microphytobenthos was extracted from the surface sediment at the sites according to Kuwae *et al.* (2008). Samples were spread on a tray to c. 5 mm depth, a nylon screen (65 μm mesh) laid over the sediment, and pre-combusted glass wool placed over the screen. The tray was kept moist by spraying with filtered seawater (0.45 μm filter pore size) and left in darkness at ambient temperature overnight. The glass wool containing migrated microphytobenthos was removed and kept dry until stable isotope analysis.

Video images

Using the methods of Kuwae (2007), we quantified foraging action rate and prey capture using a video image system at the sites. The technique affords various advantages over conventional focal observation techniques, including re-analysis and high magnification capability. Briefly, we used a digital camcorder (XL1S; Canon, Tokyo, Japan) with a telephoto zoom lens (EF 100–400 mm f/4.5–5.6L IS USM; Canon) or a mono-focal telephoto lens (EF 400 mm f/2.8L IS USM, Canon) through a lens adaptor (EF Adaptor XL; Canon). We used a focus length extension lens (Extender EF 1.4 × II or EF 2.0 × II; Canon) as required for high magnification. Video images with a focal length of 720–5760 mm (20.6–164.6 × optical magnification) were achieved by this system, making it possible to detect micro-invertebrates. We replayed and analysed the recorded video images taken at the sites (see Table S1) using slow-motion and stop-motion replay modes to analyse the rapid feeding actions of sandpipers, which are usually completed within 0.3 s.

Stable isotope analyses

Droppings from the sandpipers were pre-treated prior to stable isotope analyses, to remove isotopically fractionated metabolites, such as urea and ammonium, as well as carbonates (Kuwae *et al.* 2008). Isotope fractionation in catabolism occurs when nitrogen in amino acid is deaminated to produce metabolites depleted in ^{15}N (Fry 2006). Thus, uric acid, which may be a major nitrogen metabolite in droppings, is not fractionated because uric acid is not produced through deamination. Stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) and total organic carbon (TOC) content were measured using a Delta Plus Advantage mass spectrometer (Thermo Electron, Bremen, Germany) coupled with an elemental analyser (Flash EA 1112; Thermo Electron). Stable isotope ratios are expressed in δ notation as the deviation from standards in parts per thousand (‰) according to the following equation:

$$\delta^{13}C, \delta^{15}N = [R_{sample}/R_{standard} - 1] \times 10^3$$

where R is $^{13}C/^{12}C$ or $^{15}N/^{14}N$. PeeDee Belemnite and atmospheric nitrogen were used as the isotope standards of carbon and nitrogen, respectively. The analytical precision in the Delta Plus Advantage mass spectrometer system based on the standard deviation of the internal reference replicates was < 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$. Trophic position was calculated separately for the two basal sources (microphytobenthos and surface sedimentary detritus) using the $\delta^{15}N$ values to perform the sensitivity analysis and estimated assuming enrichment of $\delta^{15}N$ by 3.4‰ per trophic level (Post 2002). Relationships between the contribution of biofilm to shorebird total diet and trophic position were examined using Spearman's rank correlation test.

Diet assessment

Contribution of potential food sources to the diet of the sandpipers was evaluated using a three-source mixing model with the computer program IsoError (Phillips & Gregg 2001). Samples of bird droppings were chosen to represent the isotopic signature of sandpiper diets (Kuwae *et al.* 2008) because droppings offer two major advantages, i.e. no fractionation and the short turnover time necessary for migratory bird samples, over tissue samples (e.g. muscle, liver and blood), which is the more common method for diet analysis (Dietz *et al.* 2010). As fractionation occurs during the making or breaking of bonds of small molecules, we might not expect fractionation during food assimilation, i.e. uptake of large molecules, without breaking nitrogen bonds (Fry 2006). Thus, while tissue samples have significant variability and uncertainty related to fractionation factors (body conditions such as fasting), dropping samples do not. Therefore, we can be confident that undigested organic matter is defecated as droppings, but such undigested organic matter will show the same isotopic signature as assimilated organic matter. Furthermore, the use of dropping samples enabled identification of the diet of these short stopover migrants (c. 2–3 days) (Ydenberg *et al.* 2004). The baseline (^{15}N value of primary producers) is well understood to have strong locality (Fry 2006). Hence, the isotopic values of tissues would mostly reflect diets from previous stopover sites during migration, considering their longer turnover time of carbon and nitrogen in the body (weeks or even months) (Fry 2006) and the length of stay of sandpipers. Clearly, tissues introduce a 'carry over effect' (Dietz *et al.* 2010), compromising interpretation of the samples. An additional advantage of droppings, as opposed to tissues, is that no killing and/or damage to wild birds was involved in collecting samples. Although some cases showed that the stable isotope signatures

of dropping samples in the present study and the whole blood samples taken at the same site by others (Evans Ogden *et al.* 2005) are comparable, in the case of multiple food sources, droppings are likely to be enriched in relatively indigestible food sources, when compared with stomach contents or assimilated materials (Sponheimer *et al.* 2003; Kuwae *et al.* 2008). As biofilm is composed of highly digestible organic matter such as microbes and extracellular polymeric substances (Characklis & Marshall 1989), using droppings to represent diet provides a conservative estimate of the contribution of biofilm to shorebird diets. Following confirmation of homoscedasticity and normality of errors, one-way ANOVA was used to test a difference in the average contribution of biofilm to total diet between two groups (muddy flats vs. sandy flats).

Energy budget

To assess biofilm contribution to diet, we constructed an energy budget model. Major parameters for energy intake rate should include behavioural trait (foraging action rate), morphological trait (scraping biofilm mass per foraging action), and food source availability (biofilm density, assimilation efficiency and foraging time), whereas parameters for energy requirement (metabolic rate) should include morphological trait (body mass) and migratory status (energy deposition). Using these parameters, we estimated the contribution of biofilm to daily energy requirement. Biofilm water content was calculated as the difference between biofilm mass before and after drying at 60 °C for 24 h. Energy content of biofilm was measured using a bomb calorimeter (CA-4PJ; Shimadzu, Kyoto, Japan). Liquid paraffin was added to ensure complete combustion. Energy intake rate from biofilm (kJ day^{-1}) was estimated by multiplying (1) observed foraging action rate (actions per min) using the video image system, defined as rate of contact of bill tip to the sediment surface, (2) scraping biofilm mass per foraging action (mg wet wt per action) (Elner *et al.* 2005), adjusted using the relationship between body mass and the tongue spine area (see Table S2), (3) water content of biofilm (%), (4) energy content of biofilm (kJ g^{-1} dry wt), (5) assimilation efficiency (%) (Castro *et al.* 1989) and (6) available foraging time during tidal exposure in daytime (h day^{-1}), multiplied by the per cent of available foraging time spent foraging (80%) (Ntiamoa-Baidu *et al.* 1998). We adopted the 75% assimilation efficiency value (Castro *et al.* 1989), given that biofilm is composed of highly digestible organic matter (Characklis & Marshall 1989). Also, assimilation efficiency in shorebirds is reported to be c. 75% for most food types and is bird species-independent (Castro *et al.* 1989). Daily energy requirement (kJ day^{-1}) was estimated to be 2.8 × basal metabolic rate (BMR) (kJ day^{-1}) (Castro *et al.* 1992). If birds were in migratory stages, daily fattening rate was added (0.4 g day^{-1}, equal to 16 kJ day^{-1}, assuming 100% fat deposition to avoid overestimation of biofilm contribution) (Warnock & Bishop 1998; Williams *et al.* 2007). The BMR was calculated using an allometric equation for non-desert environments (Tieleman & Williams 2000) and body mass (Paulson 1993).

Next, the maximum possible contribution of biofilm was calculated using the energy budget model described above to assess the sensitivity of two parameters: body mass and TOC (biofilm density). We adjusted the following three parameters in the model: foraging action rate to 240 actions per min, available foraging time to 24 h given a variety of shorebirds being able to use nocturnal foraging (Colwell 2010), and no fat deposition. We believe that 240 actions per min is the upper limit based on behavioural observations. Water content of biofilm was estimated using the observed relationship (data were taken from all the sites):

$$\text{Water content } (\%) = 40.2 \times \text{TOC } (\%)^{0.2417} \quad (r^2 = 0.913, n = 10)$$

The energy content of biofilm was calculated using the observed relationship (data were taken from all the sites):

$$\text{Energy content } (\text{J g}^{-1}\text{dry sediment}) = 392.31 \times \text{TOC } (\%) \quad (r^2 = 0.998, n = 19).$$

Feeding apparatus morphology

We examined the tongue morphology of 130 individuals from 30 species, using macro-photographs of live birds captured by a mist net on the Tori-no-Umi intertidal sandflat (38°1.8′N, 140°54.9′E), Miyagi, Japan (see Table S3). Tongue images were recorded using a digital camera (Kiss Digital N; Canon) with a zoom lens (EF-S18–55 mm f3.5–5.6 USM; Canon). Through image analyses, the presence/absence of tongue spines was established and tongue width and the spine length were measured. Tongue spine area A was assumed to be a crescent shape and calculated according to the following equations: $A = \pi RL/2$; where, R is the half of tongue width (tongue tip radius) and L is the spine length.

Detailed tongue structures were examined using scanning electron microphotographs of a dead shorebird (Red-necked Stint *C. ruficollis*) found on the Banzu intertidal sandflat (35°24.8′N, 139°53.9′E), Tokyo Bay, Japan. Stored frozen specimens for examination were thawed and fixed in 2% glutaraldehyde/0.1 M phosphate buffer, additionally fixed with 2% OsO_4/0.1 M phosphate buffer at 4 °C for 2 h. Specimens were then rinsed in 0.1 M phosphate buffer and dehydrated in ethanol. Following dehydration, the specimens were replaced in isoamyl acetate, critical point dried, coated with a layer of sublimated OsO_4 using an osmium plasma coater (OPC80N; Filgen, Nagoya, Japan) and examined under scanning electron microscopy (JEM-6320F; JEOL, Tokyo, Japan).

Phylogenetic comparative analysis

As related species cannot be assumed to be independent, the degree of phylogenetic correction required for a comparative data set was estimated using the phylogenetic generalised least squares method (Freckleton *et al.* 2002). The parameter λ estimates the most appropriate branch length transformation for a given data and phylogeny by maximum-likelihood estimation, with values ranging from 0 (phylogenetic independence) to 1 (traits evolved according to Brownian motion on the given phylogeny). The parameter λ can either be used to estimate the degree of phylogenetic dependence of a single trait or to estimate, and simultaneously correct for, phylogenetic effects among multiple traits. The λ was first estimated for tongue spine length and tongue spine area to determine if these traits showed a phylogenetic signal. The log-likelihood ratio test was used to test whether estimated values of λ differed significantly from 0 and 1 or adopted some intermediate value. Phylogenetic generalised least squares models were then applied with averaged tongue spine length/area for each species as the dependent variable and averaged body mass for each species as an explanatory variable. As the maximum-likelihood estimate of λ for a single trait may differ from the

maximum-likelihood of λ of a regression analysis involving the same trait, λ was estimated separately for each analysis to ensure that the appropriate degree of phylogenetic correction was used. We selected a linear model structure because it fitted better (lower AICc) than a quadratic model structure. The phylogenetic super-tree of shorebirds was used for the comparative analyses (Thomas *et al.* 2004). These analyses were performed in R (R Development Core Team 2011) using code written by R. P. Freckleton in the package 'caper'.

RESULTS

Sandpipers used rapid bill actions (Table 1) and a tongue tip that is functionally capable of scraping off biofilm (Fig. 1a). Surface biofilm was collected between the bill tips, occasionally the tongue stretched out from between the tips, and the bill was then raised from the sediment surface. The bill repeatedly opened and closed with accompanying throat movements and the bolus moved back and forth. Microscopy revealed dense spines on their tongue tips (Fig. 1b–d and f, but no spines in 1e). Morphological phylogenetics showed that tongue spines were polyphyletic (Fig. 2), with sandpipers, shanks and plovers possessing tongue spines (see Table S3). The modelling revealed that the extent of development of tongue spines was phylogenetically dependent (see Table S2). Opposing to allometry, more developed spines were present in small-bodied birds even after removing the effect of phylogeny (Fig. 1h and i, see Table S2).

Three food source mixing models using stable isotope signatures (see Fig. S1) showed that the contribution of biofilm to total diet varied (Fig. 3a); for example, Red-necked Stints on the Komuke mudflat showed a 50–78% (95% confidence interval) reliance on biofilm, as opposed to 0–18% for Dunlin on the Banzu sandflat. The trophic position of shorebirds was proportionally lower as the contribution of biofilm to their total diet became higher, irrespective of basal sources (Fig 3b, microphytobenthos: Spearman's rank correlation, $S = 108$, $r^2 = 0.89$, $P = 0.007$, $n = 7$; surface sedimentary detritus: $S = 104$, $r^2 = 0.77$, $P = 0.024$, $n = 7$). When study sites were categorised into two groups (muddy vs. sandy) based on total organic carbon (TOC) or energy density in the surface sediments (a proxy for biofilm density), the average contribution of biofilm to total diet was significantly higher at muddy than sandy sites (Fig. 3c; One-way ANOVA: $df = 3.8$, $F = 19.89$, $P = 0.01252$).

Estimated energy budgets (Table 1) revealed higher contributions of biofilm to total diet at higher biofilm density sites; the relationship was consistent with those from stable isotope signatures (Fig. 3a). The energy budget model explored how biofilm contribution varied with body mass and TOC (biofilm density) (Fig. 3d). First, assuming a plausible foraging action rate at 120 actions per min and available foraging time of 12 h; birds of 30 g (Western Sandpiper size) can obtain > 60% of their daily energy expenditure on muddy sediments with TOC at 2%, whereas birds of 120 g (Red Knot *C. canutus* size) obtain < 20% on the same TOC content. Next, assuming a maximum possible foraging action rate, foraging time with no fat deposition, the model predicts that > 50% of daily energy requirements can derive from biofilm when body mass < 50 g with TOC > 0.5%. However, for birds of 120 g, biofilm cannot contribute > 50% of daily energy expenditure on sandy sediments where TOC is generally < 1%.

DISCUSSION

We empirically revealed that missing trophic links between shorebirds and biofilm exist and are widespread on intertidal flat

Table 1 Estimated energy budget and the contribution of biofilm to daily energy expenditure (mean ± SE)

Site	Species	Foraging action rate (actions per min) (A)	Scraping biofilm mass* (mg wet per action) (B)	Biofilm water content (%) (C)	Biofilm energy content (kJ g^{-1} dry wt) (D)	Biofilm energy assimilation rate† (kJ per min) (E)	Available foraging time‡ (h day^{-1}) (F)	Biofilm energy assimilation rate§ (kJ day^{-1}) (G)	Daily energy expenditure (DEE)¶ (kJ day^{-1}) (H)	Contribution of biofilm to DEE** (%) (I)
Komuke mudflat Japan	RS	114 ± 5 (98)	2.6	72.7 ± 1.3 (10)	2.4 ± 0.0 (10)	0.14 ± 0.01	7.0	60 ± 3	104	58 ± 3
Furen mudflat Japan	RS	79 ± 11 (10)	2.6	71.4 ± 0.5 (10)	5.1 ± 0.1 (15)	0.22 ± 0.03	8.9	119 ± 16	104	114 ± 16
Osaka-Nanko mudflat Japan	RS	60 ± 5 (38)††	2.6	54.2 ± 0.3 (10)	0.5 ± 0.0 (5)††	0.03 ± 0.00††	15.0	19 ± 2††	104	19 ± 2††
Roberts Bank mudflat Canada	WS	123 ± 5 (168)	2.6	48.8 ± 0.3 (6)	0.8 ± 0.0 (20)	0.10 ± 0.01	10.1	61 ± 3	106	58 ± 3
Boundary Bay sandflat Canada	WS	154 ± 9 (96)	2.6	30.9 ± 0.5 (6)	0.3 ± 0.0 (12)	0.06 ± 0.00	10.8	39 ± 3	106	37 ± 3
Boundary Bay sandflat Canada	DL	122 ± 7 (120)	2.3	30.9 ± 0.5 (6)	0.3 ± 0.0 (12)	0.04 ± 0.00	9.4	21 ± 2	147	14 ± 1
Banzu sandflat Japan	DL	53 ± 4 (28)	2.3	24.6 ± 0.3 (22)	0.1 ± 0.0 (22)	0.01 ± 0.00	4.2	3 ± 0	147	2 ± 0

Sample sizes are in parenthesis. RS, Red-necked Stint; WS, Western Sandpiper; DL, Dulin.
*Estimated using Elner *et al.* (2005) and the relationship between tongue spine area and body mass (Fig. 1i).
†$E = A \times B/1000 \times (100 - C) \times D/100$.
‡Assuming that foraging time is calculated by emersion time during the day × 0.8 (see Materials and Methods).
§$G = E \times 60 \times F$.
¶Estimated by using body mass and FMR relationships (see Materials and Methods).
**$I = G/H \times 100$.
††Possible underestimation due to observations and sampling at the low biofilm density area.

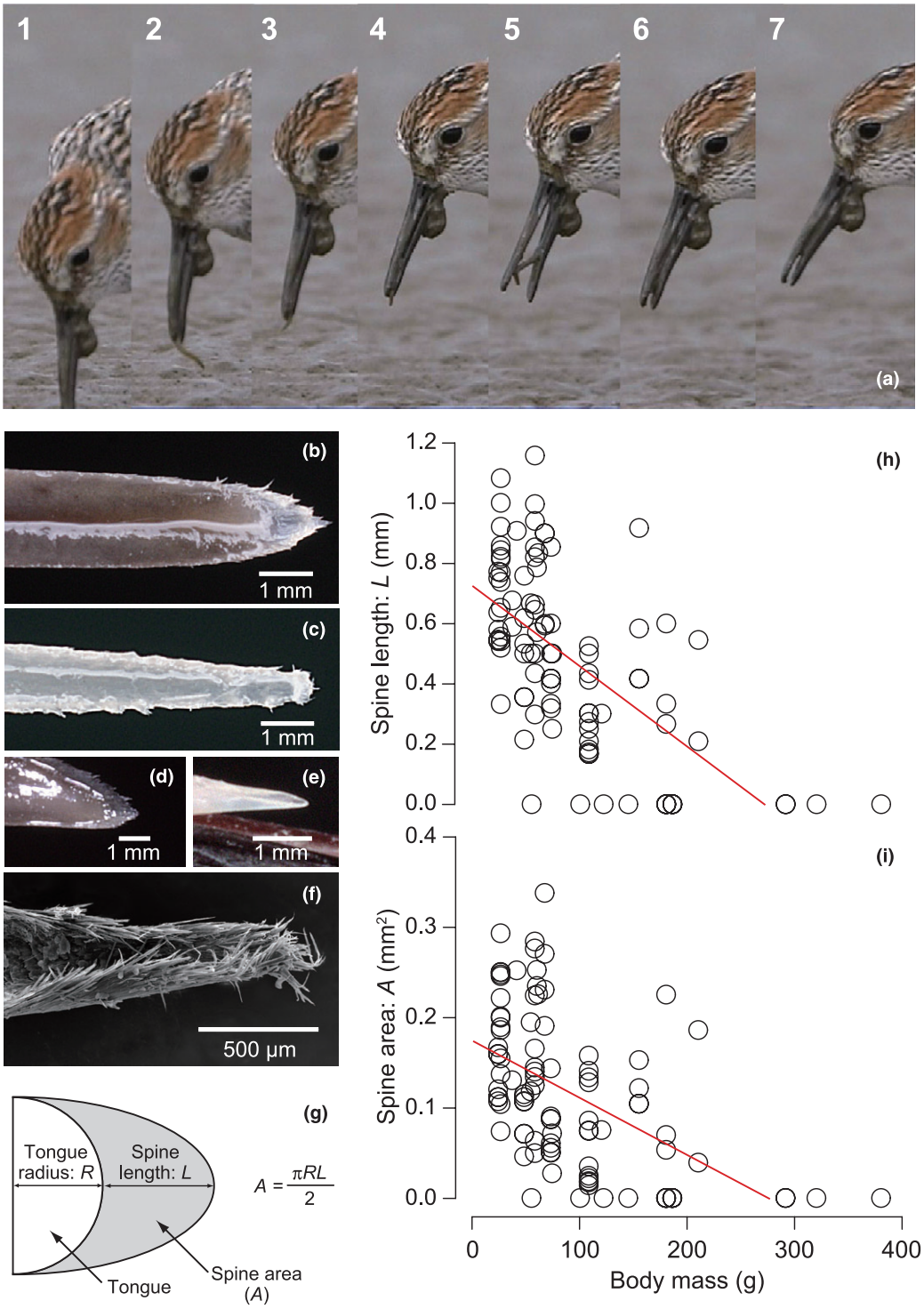

Figure 1 (a) Representative video sequence (1/30 s shot for each of the actions) of biofilm foraging behaviour (Western Sandpiper *Calidris mauri*). The actions may serve to mechanically extract food types; in this case, a mud bolus, possibly a residue of the extraction, was attached at the base of the bill. Macro-photographs of live bird tongue tips: (b) sandpiper (Sharp-tailed Sandpiper *Calidris acuminate*), (c) shank (Terek Sandpiper *Xenus cinereus*), (d) plover (Lesser Sand Plover *Charadrius mongolus*) and (e) curlew (Whimbrel *Numenius phaeopus*). (f) Scanning electron micrograph of the tongue tip of a Red-necked Stint (*Calidris ruficollis*). (h) Tongue spine length vs. shorebird body mass. (i) Tongue spine area (g) vs. shorebird body mass; the regression equations and statistics, see Table S2.

ecosystems, revising (lowering) the trophic position of these birds (Fig. 3b) and, at the community level, providing a new perspective showing greater food web complexity (Fig. 4a). Furthermore, the biofilm-shorebird trophic link appears spatially, temporally and evolutionary variable; specifically, the strength of the linkage is likely to vary dependent on the predator's trait (body mass and foraging action rate) and the environment that determines food density.

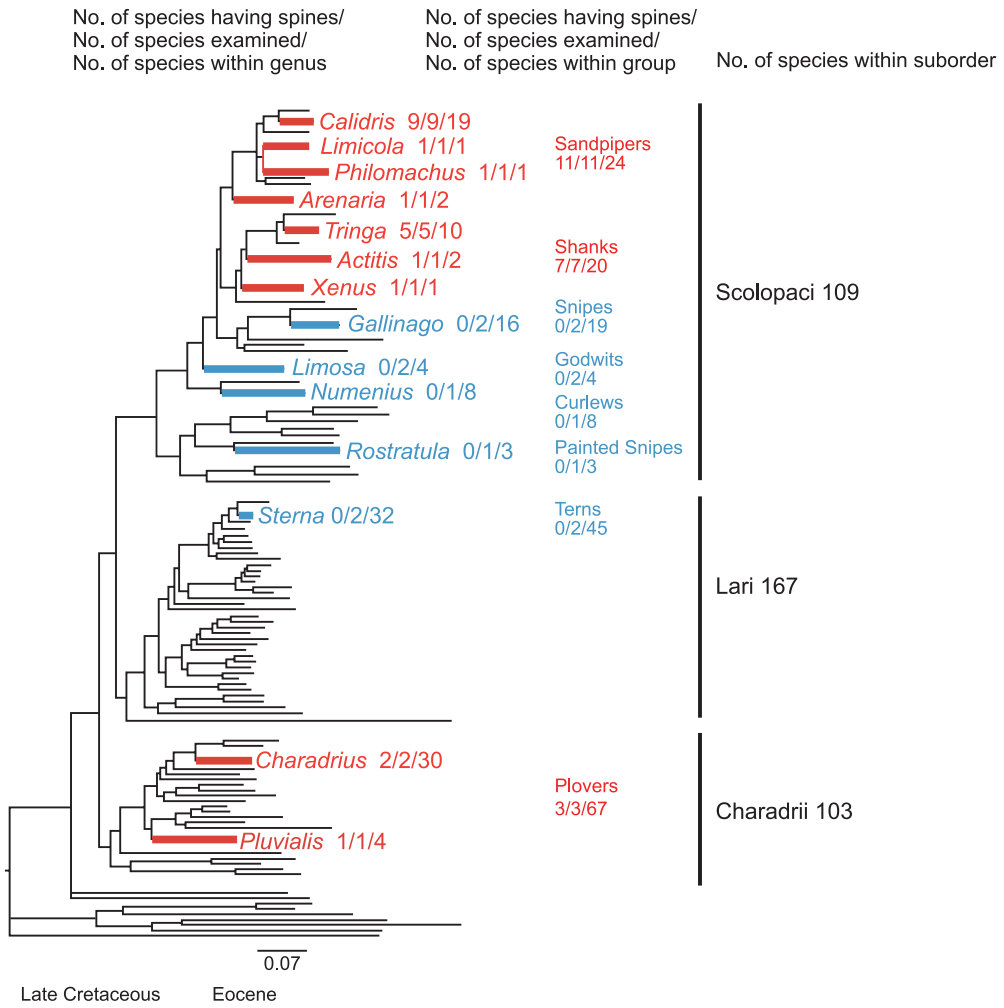

Figure 2 Phylogenetic relationship to the presence (red lineage)/absence (blue lineage) of tongue spines in different shorebird species (see Supplementary Table S3). Number of species in suborder, group, and genus are from Wetlands International (2006). Phylogenetic tree is from Baker *et al.* (2007).

The finding of a direct link between biofilm and shorebird changes the classical view where biofilm and shorebirds belonged to basal and third trophic levels, respectively, on a simple food chain (Colwell 2010; Fig 4a). In particular, in the presence of the biofilm-shorebird trophic link, the major three components of intertidal flat ecosystems, i.e. biofilm, invertebrates and shorebirds, form an intraguild predation (IGP) module (Fig. 4a). Given the fundamental change in the basic food web structure, the biofilm-shorebird links were not only 'missing' but also 'critical' links that may have major ecological consequences. A straightforward example of an expected community-level consequence is the transmission speed of interspecific effects between biofilm and shorebirds. Theory predicts that interspecific effects are, in general, more rapidly transmitted when direct than when indirect (Yodzis 1989). Thus, the direct interaction between shorebirds and biofilm implies that shorebird population dynamics may respond more quickly to the environmental factors (e.g. sediment grain size and hydrodynamic forcing) that determine the dynamics of biofilm density than previously thought. Similarly, the dynamics of biofilm density would be more rapidly affected by factors (e.g. predators and shorebirds' prey density except for biofilm) that determine shorebird dynamics than previously thought.

The more rapid transmission of interspecific effects, however, does not necessarily mean that shorebirds and biofim are more sensitive to environmental changes. Indeed, ecological theory provides several lines of reasoning that the biofilm-shorebird IGP link stabilises the three-species community. First, the IGP link is predicted to weaken trophic cascading effects (Bascompte *et al.* 2005) and support a more persistent coexistence of basal species (biofilm), consumers (invertebrates) and predators (birds). In turn, this poses that a decline in the strength of the IGP could enhance trophic cascades and result in trophic degrading (Estes *et al.* 2011). Second, theory predicts that the stability of complex ecosystems depends on the heterogeneity of distinct energy channels, their differential dynamic productivity and turnover (fast: biofilm, slow: invertebrates), and the mobile (Rooney *et al.* 2006) or adaptive (McCann & Hastings 1997; Kondoh 2003; Valdovinos *et al.* 2010) predators (birds) feeding on more abundant prey. As the prerequisite of the theory is upheld by the existence of the biofilm-bird linkage, the missing link may be a key for stabilising the real food webs. In these contexts, worldwide declines in shorebird (i.e. mobile predator) populations raise an alarm for far-reaching effects on the stability of whole ecosystems (Wetlands International 2006; Estes *et al.* 2011).

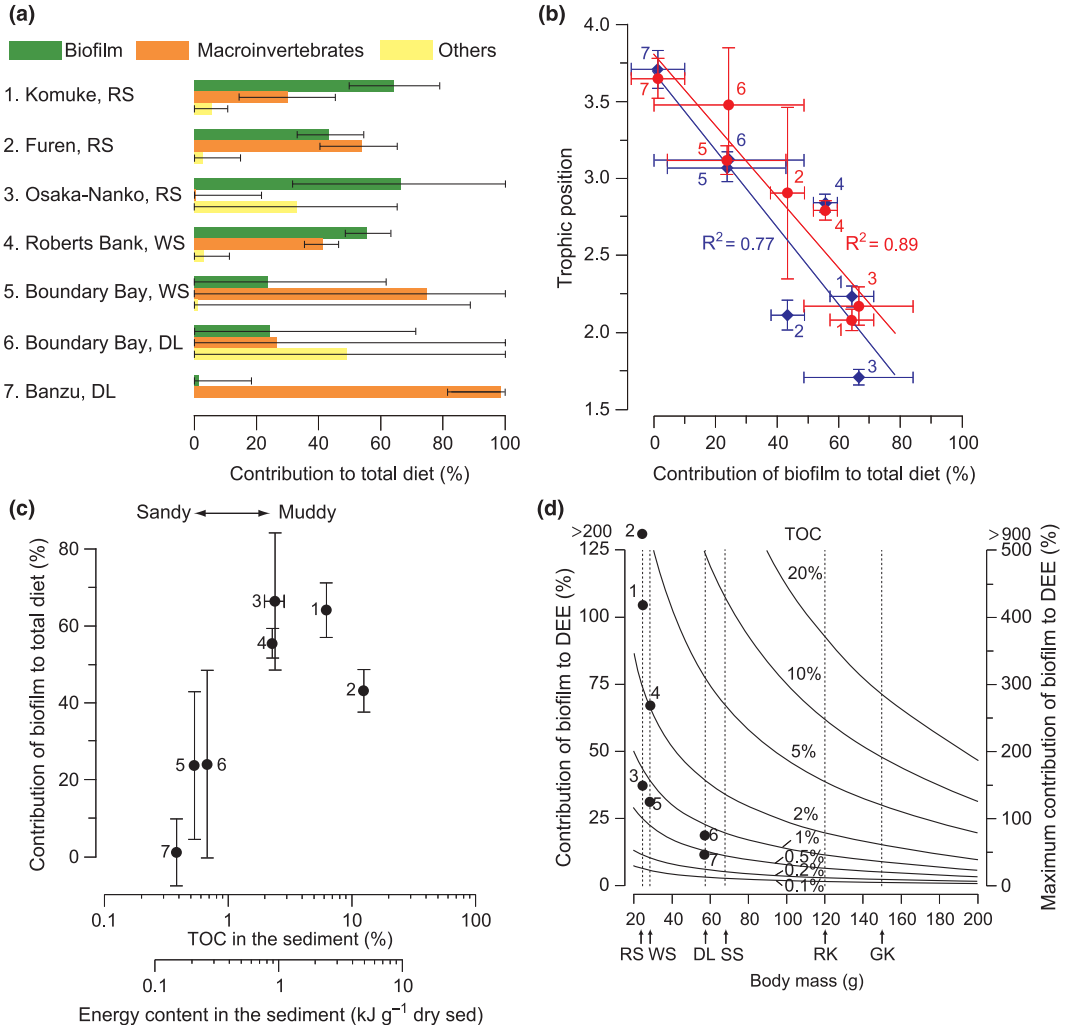

Figure 3 (a) Contribution of food sources to the diet of sandpipers. Error bars: 95% CI. (b) Trophic position of sandpipers vs. the contribution of biofilm to total diet. Basal source: microphytobenthos (red) and surface sedimentary detritus (blue). Error bars: SE. (c) Contribution of biofilm to total diet vs. total organic carbon (TOC) and energy content in the surface sediment. Error bars: SE. Horizontal error bars are for TOC. (d) Energetic model for a plausible (left axis) and the maximum possible (right axis) contribution of biofilm to daily energy expenditure. Closed circles are estimated values using the observed and measured variables at the sites. RS: Red-necked Stint; WS: Western Sandpiper; DL: Dunlin; SS: Sharp-tailed Sandpiper; RK: Red Knot; and GK: Great Knot.

Our analysis indicates that the strength of biofilm-shorebird interaction is spatially and temporally variable, depending on shorebird traits and environmental conditions. The food source mixing models from stable isotope signatures and the estimated energy budgets showed comparable values of shorebird reliance on biofilm, which was higher at high biofilm density muddy sites. Such is consistent with behavioural evidence from elsewhere (Kuwae *et al.* 2008, 2010) and a new conservation paradigm regarding the importance of mudflat habitat for producing biofilm and feeding opportunities for sandpipers that exhibit omnivory (Amano *et al.* 2010). Furthermore, shorebird reliance on biofilm is predicted to be high when the bird's body mass is small, based on energy budgets. High reliance on biofilm at high biofilm density and small body size indicates that smaller birds in conjunction with the higher energy content on muddy sediments are energetically capable of being biofilm monophagous. However, the contribution of biofilm to total diet peaked at approximately 70% of maximal, indicating that biofilm is a major, but not necessarily exclusive food source. The situation may result from, variously, foraging patch and mode choice changes in response to changes in prey availability and constraints that vary with environmental conditions (i.e. tidal stage) (Stephens & Krebs 1986; Kuwae *et al.* 2010), nutritional balance and limitation (Raubenheimer & Simpson 1997) and diet preference (Parsons *et al.* 1994).

Understanding evolutionary and constraint aspects of trait is important because consequences of ecological interactions among species are determined by their evolutionary histories, and this feeds back to influence evolutionary processes of diversification and adaptation. Our morphological phylogenetics indicates that the extent of development of tongue spines is phylogenetically dependent, suggesting that biofilm reliance would be also phylogenetically dependent. Furthermore, tongue spine possessing clades (groups of sandpipers, shanks and plovers) have greater species richness than other sister clades of Charadriiformes (Fig. 2). Also, only these former clades commonly exhibit substrate pecking behaviour, in contrast with the sister clades that do not usually peck for surficial prey items (Colwell 2010). These two lines of evidence indicate that although the role of tongue spines is not limited to biofilm scraping (McLelland 1979), the evolution of the trait can be a consequence of an adaptation

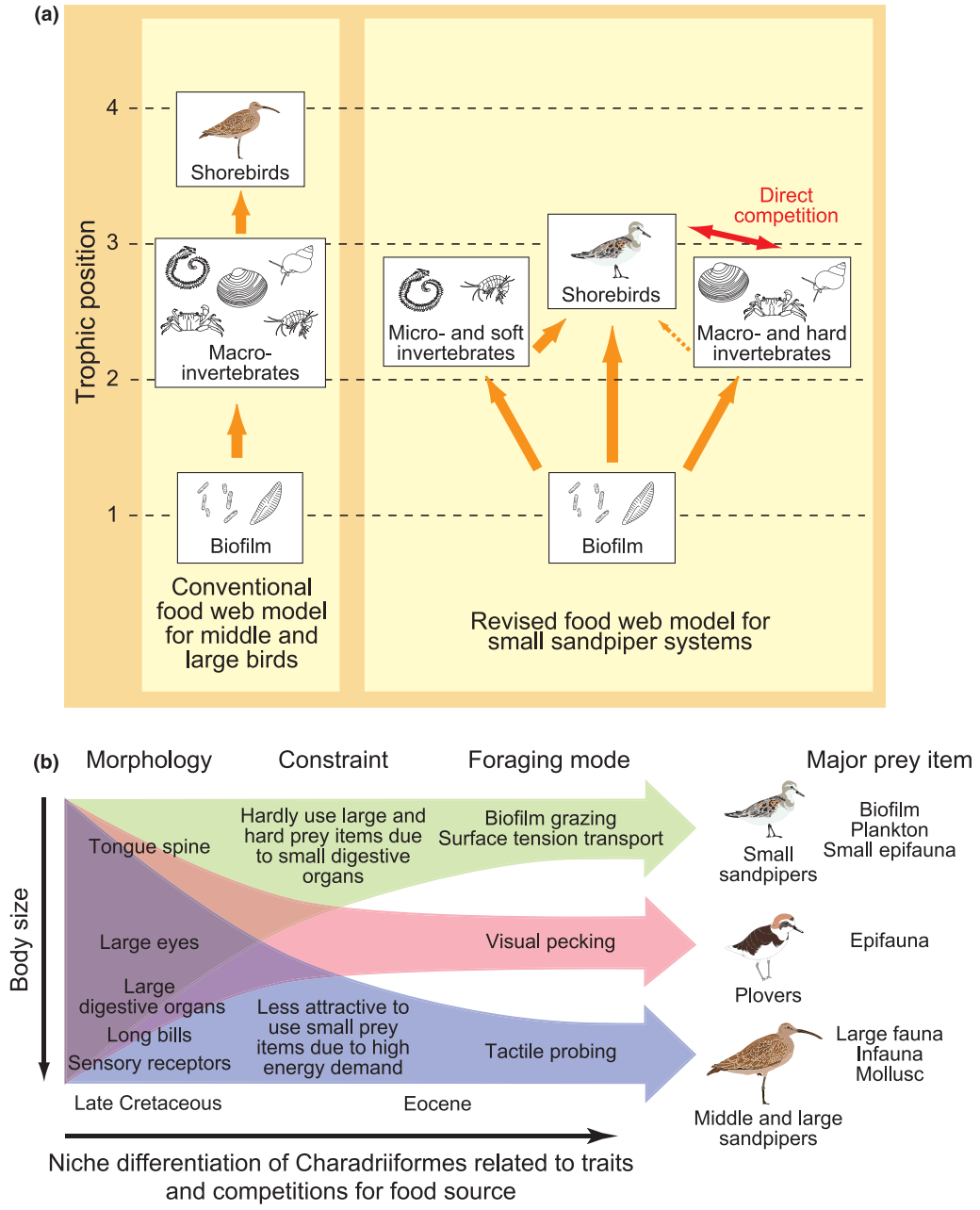

Figure 4 (a) Biofilm feeding sandpipers lead to revisions of the trophic position of the bird being lower, greater complexity of the food webs than previously thought, i.e. intraguild predation of micro- and soft-invertebrates, and direct competition with biofilm feeding macro- and hard-invertebrates. The trophic position is according to Fig. 3b. (b) Groups of closely related species share some traits by common ancestry; however, during adaptive radiations, groups of sympatric species are diversified with concomitant differentiation in traits related to their use of food sources. Within such traits, body-size and associated action rates, feeding apparatus size and digestive organ size may be key drivers for shorebirds' diversification of foraging modes, leading to niche differentiation.

responsible for exploring new niche space (diet) (Schluter 2000) and reducing extinction rate (Owens *et al.* 1999). Here, we document biofilm feeding in sandpipers possessing tongue spine; however, considering that the extent of development of tongue spines is phylogenetically constrained, future work should empirically investigate biofilm feeding in other spine possessing shorebirds, such as shanks and plovers.

The discovery of biofilm as a major food source for small sandpiper species generates a contradiction to the functional morphology adaptation hypothesis, because the narrow tubular bills of Scolopacidae species are considered adapted to exploit infaunal prey (Colwell 2010). However, given the results of the body-size dependent feeding apparatus trait and phylogenetic analyses, we propose a new conceptual model of body-size based diversification as a result of adaptive radiation for feeding (Fig. 4b). Groups share some traits (e.g. tongue spines, large eyes and long bills) through common ancestry. During adaptive radiation, sympatric species are diversified with concomitant differentiation in traits related to their use of food sources. Within such traits, body-size *per se* and associated action rates, feeding apparatus and digestive organ sizes may be key drivers for shorebird diversification of

foraging modes, leading to niche differentiation. Body size based scaling can be applied across multiple levels of biological organisation such as species and sex (Carnicer *et al.* 2009). Evolutionary and phylogenetic indications are that Scolopacidae species differentiated from the same ancestor of Charadriiformes and evolved to access larger prey in deeper sediments, whereas plovers specialised on surface prey (Colwell 2010). We argue that Scolopacidae are further differentiated because small-bodied birds were thwarted by larger, harder prey, due to the constraints of digestive organ size (van Gils *et al.* 2003), and switched to smaller, softer foods, such as biofilm. Thus, although the shorter bills and smaller digestive organs of small sandpipers may appear a disadvantage, they are compensated for by biofilm feeding. Our diversification model for feeding shows averaged situations built on body size, but adaptive foraging (Stephens & Krebs 1986; Valdovinos *et al.* 2010) can facilitate sharing of prey items between different sized birds in limited temporal and spatial scales. Nevertheless, our findings close a gap in niche space for shorebirds and reveal a wider food source spectrum. Size (small sandpipers < 20 g to large curlews > 800 g) and feeding morphology variations within shorebirds are among the most diverse of any avian group (Colwell 2010) and may be the basis for their diverse niche differentiations.

The new trophic links between birds and biofilm can help explain the macro-scale distribution and population dynamics of small-bodied sandpipers (< 30 g in body mass); including, why small-bodied sandpipers are less abundant in the African-Eurasian Flyways than other flyways (Wetlands International 2006). Small sandpipers compete with other biofilm grazers, such as mud snails *Hydrobia* at low-energy (calm) high elevation sites (Bocher *et al.* 2007). High densities of these snails on intertidal flats of African-Eurasian Flyways (Bocher *et al.* 2007) could indicate strong biofilm grazing pressure and direct competition, with a consequential negative effect on the sandpipers (Fig. 4a). Furthermore, small sandpipers hardly provide top-down control of the snails due to their limited digestion trait (Fig. 4b). However, there would be no negative effect for medium-bodied sandpipers, such as Red Knots, which utilise snails as food because of specialised digestive traits for hard shelled prey (van Gils *et al.* 2003, 2005).

Finally, we contend that exploring missing links and merging empirical and theoretical works can disentangle true network structure and dynamics. Theoretical study can further incorporate empirical data for species traits and link strengths to simulate a real world context, as well as statistically and computationally identify missing and spurious links (Clauset *et al.* 2008). In particular, sensitivity analyses of the structure and dynamics, with and without the missing links, may be useful to explore the mechanism of complex and stable networks in the real world (Bascompte 2010). In turn, empirical studies can further focus on ecological networks, because the current situation is often dominated by theoretical modelling. For example, empirical study can further contribute to network studies by quantifying the strength of actual trophic links by stable isotope and energy budget analyses, as well as quantifying regulating determinants of the strength, the properties of nodes (traits), and their variability in temporal and spatial scales.

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AUTHORSHIP

T.K. designed the research programme; T.K., E.M., S.H., K.I., J.H., T.M., and R.C.Y. performed the research; T.K. and T.A. analysed data; and T.K., M.K., and R.W.E. wrote the article.

REFERENCES

Amano, T., Székely, T., Koyama, K., Amano, H. & Sutherland, W.J.A. (2010). Framework for monitoring the status of populations: an example from wader populations in the East Asian-Australasian flyway. *Biol. Conserv.*, 143, 2238–2247.

Baker, A.J., Pereira, S.L. & Paton, T.A. (2007). Phylogenetic relationship and divergence times of Charadriiformes genera: multigene evidence for the Cretaceous origin of at least 14 clades of shorebirds. *Biol. Lett.*, 3, 205–209.

Bascompte, J. (2010). Structure and dynamics of ecological networks. *Science*, 329, 765–766.

Bascompte, J., Melián, C.J. & Sala, E. (2005). Interaction strength combinations and the overfishing of a marine food web. *Proc. Natl Acad. Sci. USA*, 102, 5443–5447.

Bocher, P., Piersma, T., Dekinga, A., Kraan, C., Yates, M.G., Guyot, T. *et al.* (2007). Site- and species-specific distribution patterns of molluscs at five intertidal soft-sediment areas in northwest Europe during a single winter. *Mar. Biol.*, 151, 577–594.

Carnicer, J., Jordano, P. & Melián, C. (2009). The temporal dynamics of resource use by frugivorous birds: a network approach. *Ecology*, 90, 1958–1970.

Castro, G., Stoyan, N. & Myers, J.P. (1989). Assimilation efficiency in birds – a function of taxon or food type. *Comp. Biochem. Physiol.*, 92A, 271–278.

Castro, G., Myers, J.P. & Ricklefs, R.E. (1992). Ecology and energetics of Sanderlings migrating to four latitudes. *Ecology*, 73, 833–844.

Cattin, M.F., Bersier, L.F., Banasek-Richter, C., Baltensperger, M. & Gabriel, J.P. (2004). Phylogenetic constraints and adaptation explain food-webs structure. *Nature*, 427, 835–839.

Characklis, W.G. & Marshall, K.C. (1989). *Biofilms*. John Wiley & Sons, New York.

Clauset, A., Moore, C. & Newman, M.E.J. (2008). Hierarchical structure and the prediction of missing links in networks. *Nature*, 453, 98–101.

Colwell, M.A. (2010). *Shorebird Ecology, Conservation, and Management*. University of California Press, Berkeley, California.

Dietz, M.W., Spaans, B., Dekinga, A., Klaassen, M., Korthals, H., van Leeuwen, C. *et al.* (2010). Do red knots (*Calidris canutus islandica*) routinely skip Iceland during southward migration? *Condor*, 112, 48–55.

Elner, R.W., Beninger, P.G., Jackson, D.L. & Potter, T.M. (2005). Evidence of a new feeding mode in Western Sandpiper (*Calidris mauri*) and Dunlin (*Calidris alpina*) based on bill and tongue morphology and ultrastructure. *Mar. Biol.*, 146, 1223–1234.

Estes, J.A. *et al.* (2011). Trophic downgrading of planet earth. *Science*, 333, 301–306.

Evans Ogden, L.J., Hobson, K.A., Lank, D.B. & Bittman, S. (2005). Stable isotope analysis reveals that agricultural habitat provides an important dietary component for nonbreeding dunlin. *Avi. Conserv. Ecol.*, 1(1), 3. URL: http://www.ace-eco.org/vol1/iss1/art3/

Freckleton, R.P., Harvey, P.H. & Pagel, M. (2002). Phylogenetic analysis and comparative data: a test and review of evidence. *Am. Nat.*, 160, 712–726.

Fry, B. (2006). *Stable Isotope Ecology*. Springer, New York.

van Gils, J.A., Piersma, T., Dekinga, A. & Dietz, M.W. (2003). Cost-benefit analysis of mollusc-eating in a shorebird. II. Optimizing gizzard size in the face of seasonal demands. *J. Exp. Biol.*, 206, 3369–3380.

van Gils, J.A., de Rooij, S.R., van Belle, J., van der Meer, J., Dekinga, A., Piersma, T. *et al.* (2005). Digestive bottleneck affects foraging decisions in red knots *Calidris canutus*. *I. Prey choice. J.Anim. Ecol.*, 74, 105–119.

Ings, T.C., Montoya, J.M., Bascompte, J., Blüthgen, N., Brown, L., Dormann, C.F. *et al.* (2009). Ecological networks – beyond food webs. *J. Anim. Ecol.*, 78, 253–269.

Kondoh, M. (2003). Foraging adaptation and the relationship between food-web complexity and stability. *Science*, 299, 1388–1391.

Kuwae, T. (2007). Diurnal and nocturnal feeding rate in Kentish plovers *Charadrius alexandrinus* on an intertidal flat as recorded by telescopic systems. *Mar. Biol.*, 151, 663–673.

Kuwae, T., Beninger, P.G., Decottignies, P., Mathot, K.J., Lund, D.R. & Elner, R.W. (2008). Biofilm grazing in a higher vertebrate: the Western Sandpiper, *Calidris mauri*. *Ecology*, 89, 599–606.

Kuwae, T., Miyoshi, E., Sassa, S. & Watabe, Y. (2010). Foraging mode shift in varying environmental conditions by dunlin *Calidris alpina*. *Mar. Ecol. Prog. Ser.*, 406, 281–289.

Mathot, K.J., Lund, D.R. & Elner, R.W. (2010). Sediment in stomach contents of Western Sandpipers and Dunlin provide evidence of biofilm feeding. *Waterbirds*, 33, 300–306.

McCann, K. & Hastings, A. (1997). Re-evaluating the omnivory-stability relationship in food webs. *Proc. R. Soc. Lond. B*, 264, 1249–1254.

McLelland, J. (1979). Digestive system. In: *Form and Function in Birds* (eds. King, A.S. & McLelland, J.). Academic Press, London, Vol. 1, pp. 69–90.

Ntiamoa-Baidu, Y., Piersma, T., Wiersma, P., Poot, M., Battley, P. & Gordon, C. (1998). Water depth selection, daily feeding routines and diets of waterbirds in coastal lagoons in Ghana. *Ibis*, 140, 89–103.

Owens, I.P.F., Bennett, P.M. & Harvey, P.H. (1999). Species richness among birds: body size, life history, sexual selection or ecology? *Proc. R. Soc. Ser. B.*, 266, 933–939.

Parsons, A.J., Newman, J.A., Penning, P.D., Harvey, A. & Orr, R.J. (1994). Diet preference of sheep: effect of recent diet, physiological state, and species abundance. *J. Anim. Ecol.*, 63, 465–478.

Paulson, D.R. (1993). *Shorebirds of the Pacific Northwest*. University of Washington Press, Seattle.

Petchey, O.L., Beckerman, A.P., Riede, J.O. & Warren, P.H. (2008). Size, foraging, and food web structure. *Proc. Natl Acad. Sci. USA*, 105, 4191–4196.

Phillips, D.L. & Gregg, J.W. (2001). Uncertainty in source partitioning using stable isotopes. *Oecologia*, 127, 171–179.

Post, D.M. (2002). Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology*, 83, 703–718.

R Development Core Team. (2011). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria, ISBN: 3-900051-07-0, URL. <http://www.R-project.org>.

Raubenheimer, D. & Simpson, S.J. (1997). Integrative models of nutrient balancing: application to insects and vertebrates. *Nutr. Res. Rev.*, 10, 151–179.

Rooney, N., McCann, K., Gellner, G. & Moore, J.C. (2006). Structural asymmetry and the stability of diverse food webs. *Nature*, 442, 265–269.

Schluter, D. (2000). *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford, UK.

Sponheimer, M. *et al.* (2003). An experimental study of carbon-isotope fractionation between diet, hair, and feces of mammalian herbivores. *Can. J. Zool.*, 81, 871–876.

Stephens, D.W. & Krebs, J.R. (1986). *Foraging Theory*. Princeton University Press, New Jersey.

Thomas, G.H., Wills, M.A. & Székely, T. (2004). A supertree approach to shorebird phylogeny. *BMC Evol. Biol.*, 4, 28.

Tieleman, B.I. & Williams, J.B. (2000). The adjustment of avian metabolic rates and water fluxes to desert environments. *Physiol. Biochem. Zool.*, 73, 461–479.

Valdovinos, F.S., Ramos-Jiliberto, R., Garay-Narváez, L., Urbani, P. & Dunne, J.A. (2010). Consequences of adaptive behaviour for the structure and dynamics of food webs. *Ecol. Lett.*, 13, 1546–1559.

Warnock, N. & Bishop, M.A. (1998). Spring stopover ecology of migrant Western Sandpipers. *Condor*, 100, 456–467.

Wetlands International. (2006). *Waterbird Population Estimates*, 4th edn. Wetlands International. Wageningen, the Netherlands.

Williams, T.D., Warnock, N., Takekawa, J.Y. & Bishop, M.A. (2007). Flyway-scale variation in plasma triglyceride levels as an index of refueling rate in spring-migrating Western Sandpipers (*Calidris mauri*). *Auk*, 124, 886–897.

Ydenberg, R.C., Butler, R.W., Lank, D.B., Smith, B.D. & Ireland, J. (2004). Western sandpipers have altered migration tactics as peregrine falcon populations have recovered. *Proc. R. Soc. Lond. B*, 271, 1263–1269.

Yodzis, P. (1989). *Introduction to Theoretical Ecology*, Harper and Row, New York.

Zwarts, L., Blomert, A.M., Ens, B.J., Hupkes, R. & van Spanje, T.M. (1990). Why do waders reach high feeding densities on the intertidal flats of the Banc d'Arguin, Mauritania? *Ardea*, 78, 39–52.

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