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Expression of Annual Cycles in Preen Wax Composition in Red Knots: Constraints on the Changing Phenotype

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ABSTRACT

Birds living in seasonal environments change physiology and behavior in correspondence to temporally changing environmental supplies, demands and opportunities. We recently reported that the chemical composition of uropygial gland secretions of sandpipers (Scolopacidae, order Charadriiformes) changes during the breeding season from mixtures of monoesters to diesters, which fulfill specific functions related to incubation. A proper temporal match between the expression of diester preen waxes and incubation requires a flexible organization of the trait. Here we analyze the possible degrees of flexibility with reference to the functionality of better-understood molt and body mass cycles of free-living and captive red knots (Calidris canutus). The relative flexibility of seasonal cycles in preen wax composition was examined by two experimental perturbations: (1) giving birds restricted access to food and (2) monitoring them long-term under a constant photoperiodic regime. We found that wax type cannot change instantaneously, but that changing the type of wax is under similar organizational time constraints as the replacement of feathers. Just as molt and mass cycles, the seasonal rhythm of diester secretion appeared to be under endogenous control: most birds placed in a constant photoperiod still maintained seasonally changing preen waxes. Diester preen wax secretion was synchronized with the peak in body mass in spring, but became less well expressed under constant photoperiodic conditions and when food availability was limited. J. Exp. Zool. 307A:127–139, 2007. © 2007 Wiley-Liss, Inc.

of monoesters secreted during the non-breeding season is replaced by higher molecular weight diesters in female mallards (Anas platyrhynchos; Kolattukudy et al., '87) and in many species of sandpiper (Scolopacidae; Reneerkens et al., 2002a). For red knots there is evidence that diesters are less smelly than monoesters. Such “olfactory crypsis” is particularly important for birds that incubate a clutch in nests that are fully exposed to mammalian predators (Reneerkens et al., 2005). The fact that diester preen waxes are not secreted year-round suggests that there are (energetical and/or functional) costs associated with diester production—secretion and/or—application, which are outweighed by their benefits only during incubation (Reneerkens et al., 2005).

If diester preen waxes are costly to make and important for “olfactory crypsis” during incubation, but not during other life cycle stages, and if preen wax composition can be changed instantaneously, diester preen waxes should not be secreted until the day at which incubation starts. When red knots skip a breeding season they should forego producing diester preen waxes; when they lose a clutch to a predator, preen wax composition should instantaneously change to monoesters.

Many seasonal changes in phenotype of migratory birds are endogenously controlled (Gwinner, '86, '90). Endogenous regulation of phenotypic traits is adaptive when it takes a relatively long time to change from one phenotypic state to another state. The phenotypic change should then be initiated before the changed phenotype is actually needed. Complete wing feather replacement in shorebirds such as red knots lasts 60–100 days (Boere, '76), and is endogenously controlled (Cadée et al., '96; Piersma, 2002b). A complete shift in preen wax composition from monoesters to diesters at the level of a population seems to happen within 3–6 days (Reneerkens et al., 2002a). Red knots secrete three distinct preen wax types during an annual cycle (Dekker et al., 2000; Sinninghe Damsté et al., 2000) all of which consist of esters, which are alcohols condensed to fatty acids. Because the chemical make-up of the three preen wax types is essentially similar, we expect that an instantaneous shift from one to another type of preen wax mixture is possible as long as the required fatty acids and alcohols are available. However, if changing the production from monoester to diester preen waxes would entail considerable modifications of the biochemical/metabolic pathways, the changes would need to be pre-arranged in time. Just like molt and pre-migratory fueling, the organization would then be more in need of some sort of endogenous control.

In examining the organization of seasonal changes in preen wax, we will focus on the shift from monoesters to diesters because studies of its function clearly predict these shifts to best occur instantaneously. We examined flexibility in shifts to diester preen wax secretion with reference to the better-understood annual cycles in molt and pre-migratory fueling in free-living and captive birds. To tease apart the expression of the different phenotypic traits, annual cycles were perturbed by either limiting the daily food resources or by placing birds in a constant photoperiodic regime. Under the assumption that a change from mono- to diester preen waxes at the start of incubation best occurs overnight, we predicted (1) that red knots in captivity would stop producing (costly) diester preen waxes (as they do not breed in captivity) and (2) birds in the treatment groups would first stop expressing diester preen waxes before “giving up” molt and body mass cycles.

**METHODS**

**Birds**

In total, 206 preen wax samples (including the 159 by Reneerkens et al., 2002a) of wild red knots were collected in several different months in the year at different locations worldwide, to get a complete overview of preen wax composition year-round. Based on plumage characteristics (Prater et al., '77), we know that all birds in our analysis were in their second calendar year or older.

Twenty-seven red knots of the subspecies islandica were caught with mistnets at night at high tide roosts in the Dutch Wadden Sea between October 1995 and August 2000. The birds were brought into captivity at NIOZ, Texel, within 100 km from their catch sites. The birds were maintained in five separate outdoor aviaries where they experienced the local light regime and ambient temperatures. Eleven birds in two of the aviaries were food restricted between March 6, 2002 and July 9, 2002. During this period, the birds daily obtained ad libitum trout pellets for 6 hr (between 10 AM and 4 PM) only, which was enough for birds to maintain and slowly increase body mass, but not with a similar fueling rate as captive Red Knots that had access to food for 24 hr/d during pre-migratory fueling (Reneerkens et al., 2002b). A different group of 14 red knots

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were caught in October and November 1994 (except for one bird that was caught in October 1995) and kept in outdoor aviaries until November 26, 1996, when they were placed in two separate indoor aviaries with a constant photoperiod of 12 hr:12 hr light–dark (L:D) cycle. As seasonal changes in photoperiod are used by many animals to time seasonal events (Gwinner, '86, '90), placing birds in a constant photoperiod was expected to perturb the temporal organization of seasonal changes of the phenotype. Air temperature was kept constant at 15°C in the indoor facilities with occasional daily peaks of 25°C in summer (June–September). All red knots were individually banded with a numbered metal ring.

All aviaries measured 2 m × 4 m with a height of approximately 2 m and contained a small area of “mudflat” with continuously flowing seawater. The hard surface of the aviaries was kept wet with seawater also. A through flow freshwater basin was used by the birds to bath and drink (see Piersma and Ramenofsky, '98 for a photograph of such an aviary). Birds were fed protein-rich trout pellets that were always available ad libitum. All red knots were older than 2 years at the start of the experiment. Each aviary, indoor and outdoor, contained both male and female red knots, but females were in minority in each treatment group (sex ratios of 10:5, 8:3 and 10:4 males: females in the control, the food restricted and the birds in a constant photoperiodic regime, respectively). Because male and female red knots have the same timing of spring migration and because both sexes incubate, no differences in annual cycles were expected.

At weekly intervals, the birds were taken out of their aviaries. As the aviaries were cleaned and disinfected, body mass was measured at least an hour after they could last have fed. The amount of breeding plumage (from 1, complete winter plumage, to 7, complete breeding plumage) were scored. In our analyses, we determined average daily mass gain by subtracting the body mass value during the preceding measurement from the measurement following the date at which daily mass gain was determined, divided by the number of days between these measurements. The end of the mass plateau in spring was determined by selecting the first date at which birds weighed more than their average during the experiment and lost at least 14 g within the next week. Preen wax from the uropygial gland was sampled by carefully massaging the uropygial gland with a cotton bud.

All captive birds were studied for 17 months, except the birds that were food restricted that were studied for 6 months between February and August 2002 and of which preen wax samples (253 in total) were collected between May 7 and July 22, 2002. The red knots in the outdoor aviaries were studied between February 5, 2002 and July 1, 2003. In the period between June and August 2002, body mass and molt was measured and preen wax sampled on a bi-weekly basis of this group of birds, as well as of the birds that were food restricted. In total 1,210 preen wax samples were collected of the birds in outdoor aviaries that received food ad libitum throughout the experiment. The 14 birds in the invariable, indoor environment were studied from February 4, 2001 to July 4, 2002, but preen wax was only sampled for 12 months between March 6, 2001 and March 12, 2002 (616 samples in total). Red knot 485 (outdoor aviaries) died on May 24, 2003, 6 weeks before the end of the experiment, of unknown cause. The experiment was performed under auspices of the Animal Experiment Committee (DEC) of the Dutch Royal Academy of Sciences (KNAW) and conforms to NIH guidelines.

**Preen wax collection and characterization**

We used the procedures of molecular analysis of preen waxes by gas chromatography as described by Dekker et al. (2000). Briefly, preen wax was brought into solution with ethyl acetate (1 mg wax/ml ethyl acetate). The samples were injected into a gas chromatograph (Hewlett-Packard 6890 Series II, using a 25 m × 0.32 mm fused silica capillary column coated with CP-Sil 5; film thickness 0.12 μm) at 70°C, subsequently heated to 130°C at 20°C/min and then at 4°C/min to 320°C where the oven temperature was held at for 35 min.

To determine the relative abundance of the different wax mixtures in a sample, the highest peak in a pure mixture of monoesters A, monooesters B and diesters was selected. The peak of hexadecyl 2-methyloctanoate (Dekker et al., 2000) was indicative of monoesters A, the peak of octadecyl 2-hexanoate for monoesters B and for the easily recognizable mixture of diesters the highest peak in the mixture, 1,2-tetradecanediyl 1-hexadecanoate 2-octadecanoate (Sinninghe Damsté et al., 2000), was used (Fig. 1). Using the integration software, we estimated the relative surface area of each of the three selected peaks.
and used this as an estimation of the proportion of different waxes in the secretions.

RESULTS

Mass and molt under natural photoperiods

Captive red knots subjected to natural photoperiodic conditions maintain annual cycles in molt and body mass (Piersma and Ramenofsky, ’98), cycles that are comparable to those of free-living birds (Piersma et al., ’95). In May–early June they showed peaks in body mass (Fig. 2) coinciding with the natural period of northward migration to the Arctic breeding grounds. During their first 2 years in captivity, islandica red knots maintain a mass peak in winter (Piersma and Ramenofsky, ’98), but these peaks then disappear (TP personal observations) as they did in the long-term captive birds studied here.

The contour feather molt in spring began before the increase in body mass (Fig. 2). By the time the birds had obtained maximal fuel stores, at the end of June, they were in (almost) complete alternate plumage. After the birds had lost their fuel stores by fasting for several days, molt towards a basic plumage began in August. The birds also began replacing their primary wing feathers at this stage. This is typical for red knots that forego a breeding season and spend the summer in Western Europe (Boere, ’76). An individual red knot that was in a poor physical condition refrained from going into molt and did not gain body mass in anticipation of a migratory flight (4462; Fig. 2).

Seasonally changing preen wax composition of wild and captive red knots under natural photoperiods

The annual cycle in preen wax composition in wild red knots is depicted in Figure 3b. Between the end of July and mid-April, a period of approximately 9 months, wild red knots invariably secreted monooesters mixture A. This includes mainly birds in their wintering grounds but also 18 birds sampled in Brazil that were fueling up for the first part of a migratory flight to Delaware Bay (USA) at the end of April (Piersma et al., 2005). Red knots in Delaware Bay in May during the final long-distance flight to the High Arctic breeding area secreted only monooester B. From early June onwards, all red knots caught on the High Arctic breeding areas secreted mainly diester preen waxes, as has been described for 19 sandpiper species, including red knot (Reneerkens et al., 2002a). Diesters continue to be secreted during the period of incubation. After incubation, at hatch, the preen wax composition shifts back to the monooester type. Red knots sampled just after hatch in mid-July secreted preen waxes consisting of mainly diesters with fractions of either monooesters A

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or monoesters B. None of the investigated individuals in June or July secreted a pure mixture of monoesters B only. After a short transition period in July on the breeding grounds during which preen wax mixtures of diester with monoesters A or B were secreted, birds secreted a pure mixture of monoesters A for nearly the entire non-breeding period from mid-July until mid-April (Fig. 3b).

Inter-individual variation in the chemical composition of the three preen wax mixtures was not found either in captive or wild red knots. The annual cycles in preen wax composition of the captive red knots (control) were synchronized between individuals (Fig. 2) and similar to an “average annual cycle” of wild birds (Fig. 3). Monoesters A were secreted during a period with on average no changes in body mass (Fig. 4). When
the birds started to put on weight in spring, the shift to monoesters B took place. During the short period of highest weekly mass gain, birds secreted only monoesters B. During the mass plateau, when on average no weight was gained or lost, the shift in preen waxes to diester type took place (Fig. 4). After this period of stable body mass, the captive birds lost weight by fasting for several weeks. It is during this period that the captive birds secreted pure diester preen wax (Figs. 2 and 4). The captive red knot (4462) in poor physical condition did not secrete diester preen waxes and also less often secreted monoesters B than the other birds (Fig. 2).

Despite the overall similarity between the annual cycle in preen wax composition of free-living red knots and the 15 birds kept in outdoor aviaries (Fig. 3), in the captive birds monoesters A were more gradually replaced with monoesters B in approximately 9 weeks time rather than 2 weeks in the whole wild population, after which a slow shift of on average 30 days to diesters took place, rather than the 2 weeks in the wild (Fig. 3). Diester preen wax secretion in June and July persisted longer in wild red knots (4–6 weeks) than in the captive individuals (on average less than 1 week) too.
**Phenotypic flexibility of food-restricted red knots**

The birds that received only 6 hr food per day showed lower maximal body mass during the spring fueling period (*t*-test, *t*₂₄ = 8.56, *P* < 0.001; Fig. 5). Five individuals, however, still gained weight and were substantially heavier during some time when they were food-restricted than before they were food-restricted (average maximal weight gain: 22.2 g, range 16–30 g; Fig. 5) compared with six birds that did not (average maximal weight gain: 4 g, range –4 to 9 g). Contour feather molt, indicated by the change in plumage scores, also remained absent or was less well pronounced compared with birds that received food ad libitum (maximal plumage score, *t*-test, *t*₂₄ = 6.62, *P* < 0.001; only three birds showed an increase in plumage score by at least 2 units during the period of food restriction, of which two birds had a maximum plumage score of 3, and one of 5).

Six of the 11 food-restricted birds secreted diester preen waxes in spring, and only two of them secreted pure diester preen waxes for a short period (Fig. 5). Also, the shift back to monoesters A occurred earlier than in the control birds that received food ad libitum (first day after spring mass peak with more than 50% monoesters A, *t*-test, *t*₂₄ = 2.51, *P* = 0.019). The timing of pheno-

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**Fig. 5.** Body mass and plumage score of 11 red knots that were food restricted during spring 2002. The period during which birds were food restricted is indicated with a gray horizontal bar. The average development of body mass and plumage score of the red knots in the outdoor aviaries that received ad libitum food (control birds) is depicted with thick lines, as a reference. Vertical lines indicate standard errors around the average. The percentage of monoesters A (white), monoesters B (gray) and diesters (black) in preen gland secretions throughout the experiment is shown for each individual bird with stacked bars that complete 100% each. The average seasonal changes of preen wax composition of the control birds that received ad libitum food are depicted as a reference.
type changes, if these occurred, did not differ from birds that received food ad libitum, but there was less expression in the food-restricted birds (Fig. 5).

The degree to which diester preen waxes were secreted, indicated by the maximal percentage of diesters during the treatment, was not correlated with the maximal body mass \((F_{1,10} = 0.006, P = 0.94, R^2 = 0.0006)\) or plumage score \((F_{1,10} = 0.67, P = 0.43, R^2 = 0.069)\). When we ranked individuals on the basis of the amount of expression, mass peaks and pre-alternate molt, there was no correlation between the degree of expression of mass peak and molt and the maximal percentage of diesters secreted \((R_S = -0.235, P = 0.50;\) Fig. 6A).

**Phenotypic flexibility of red knots experiencing a constant photoperiod**

As shown before for canutus red knots (Cadée et al., '96), seasonal mass and molt changes of islandica red knots that had experienced a constant photoperiod for more than 4 years became free-running and deviated from natural annual cycles in these traits (Fig. 7). The annual cycles in preen wax composition of these red knots also strongly deviated from those experiencing seasonal cycles in day length (Fig. 7). The timing and duration of the body mass increases differed much from those in red knots experiencing natural photoperiods and also strongly varied between individuals (Figs. 2 and 7). Some individuals showed no obvious single mass peaks during the experiment (Fig. 7), either no peaks at all (e.g., bird 213) or an oscillating pattern in body mass (e.g., bird 189).

Cycles in molt were also perturbed by the constant photoperiodic regime but not in all individuals. Only five of the 14 individuals showed more or less “normal” cycles of plumage scores. These birds wore an alternate plumage, indicated by plumage scores of 5 or more, during peak body mass and a basic plumage (plumage score 2 or less) when body mass was low and stable. As in red knots experiencing seasonal photoperiodic regimes, wing molt occurred after the peak in body mass. Contour feather molt followed the mass peaks in some birds, just as captive red knots under natural photoperiodic conditions (e.g., individuals 161 and 295, Fig. 7). In other individuals, cycles in plumage score were perturbed. A few individuals remained in full alternate plumage throughout the period of observation (e.g., individuals 189 and 192).

Seasonal cycles in preen wax composition also differed from red knots experiencing natural photoperiods. The predominant preen wax mixture secreted during the experiment consisted of mixtures of monoesters A and B (Fig. 7), in contrast to red knots experiencing natural photoperiods that secrete pure monoester A preen waxes during most of the year (Fig. 2). Only some red knots secreted diesters, but always mixed with monoesters. The timing of diester preen wax secretion in those nine of 14 birds that did secrete diesters was linked with their peak in body mass (Figs. 7 and 8) and therefore also occurred between September and January instead of May–June and was, in contrast to the birds in natural photoperiods, not synchronized between individuals (Fig. 8). Changes in preen wax composition from monoesters to diester preen waxes persisted only in those few individuals that also showed clear cycles in body mass and molt (e.g., individual 295 and 161; Fig. 7). However, also in these individuals no pure diester preen waxes were secreted and in some individuals a clear shift to diesters was absent even though they showed clear
cycles in mass and molt (e.g., individual 178 and 220; Fig. 7). When individuals were ranked on the basis of the expression of body mass and molt, individuals with the highest scores also secreted the highest percentage of diesters ($R_S = -0.781, P < 0.002$; Fig. 6B).

**DISCUSSION**

**Constraints on wax ester shift**

Despite not being able to migrate and reproduce, control captive red knots exposed to seasonally

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**Fig. 7.** Seasonal changes in body mass (thick line), primary wing feather molt, the amount of summer plumage (thin line; score between 1 and 7) and preen wax composition in eight red knots kept in aviaries indoors with a constant photoperiod (12 hr light, 12 hr darkness) between 4 February 2001 and 4 July 2002. The percentage of monoesters A (white), monoesters B (light gray) and diesters (black) in preen gland secretions at a given date is shown for each individual bird throughout the experiment with stacked bars that complete 100% each. The eight of 14 individuals shown here represent different “robustness” of seasonal phenotypic cycles and are ordered by rank (see text for the criteria used). Assigned ranks are shown between brackets behind the number of the individual bird.
changing day lengths and with ad libitum access to food exhibited seasonal phenotype cycles that were very similar to free-living birds (confirming Piersma and Ramenofsky, '98; Piersma, 2002a). The list of seasonally changing phenotypic traits can thereby be extended with preen wax composition and we must reject our first hypothesis that captive red knots skip the expression of a trait associated with incubation.

Also in contrast to the hypothesis of instantaneous changes if no organizational costs are involved, changes from monoester to diester preen wax took place over a protracted period of time. Complete shifts from monoester to diester preen waxes in captive birds lasted on average 30 d in captive red knots, but could be accomplished in a shorter period (e.g., 13–14 days in some individuals, Fig. 2). It thus appears that, like molt

Fig. 7. Continued.
and pre-migratory fueling, changes from mono- to diester secretions cannot be accomplished overnight. Shifts from diester waxes back to monoesters A were faster and were accomplished in 3–8 days in some individuals, suggesting that the shift to diester secretion is more constrained than the shifts back to monoester waxes. A better understanding of the biochemical pathways of monoester and diester preen waxes, may yield proximate explanations for these observations. Alternatively, chemical shifts in the secretions are time consuming because the preen gland is a kind of “reservoir” of waxes, that prevents the secretions to change instantaneously.

**Endogenously regulated expression**

If the transition from one phenotypic state to another takes time, the onset of such changes should start well before the time that the new phenotype actually becomes useful. In such cases endogenous regulation of the onset of change is adaptive (Gwinner, ’86, ’90, ’95; Nelson et al., 2002). That seasonal cycles in preen wax composi-
different traits in food-restricted birds supports this view. In contrast, in red knots exposed to a constant photoperiodic regime, the expression of diester secretion was highest in individuals that still showed the most coherent phenotype cycles (Fig. 6).

Even though diester secretion changes in preen wax composition are endogenously regulated and cannot be changed as instantaneously as we predicted, this trait may still be more flexibly organized than are molt and seasonal mass changes. After all, transition times of preen wax composition from one to another state were still relatively short compared with the transition times of the other traits. It may be adaptive for animals to not completely advance seasonal cycles of traits when these are functional during reproduction only, as is the case for changes in preen wax composition from mono- to diesters. Reproduction in long-lived species like red knots does not occur every year due to predation of clutches or because bad environmental (food) conditions do not enable a migratory flight to the breeding grounds (D.I. Rogers pers. comm.). In such non-breeding years, it would be adaptive if changes of traits associated with reproduction could be turned off or not switched on at all. Phenotype changes that enhance survival, wing molt for example, should always continue and therefore be more robust.

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