Binding pattern of $^{125}$Iodine thyroxine and tri-iodothyronine in skin and liver tissues of spotted munia, *Lonchura punctulata*: Co-relation to seasonal cycles of breeding and molting

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Prevalent notion about thyroid hormones is that thyroxine ($T_4$) is a mere precursor and physiological effects of thyroid hormones are elicited by tri-iodothyronine ($T_3$) after mono-deiodination of $T_4$. Earlier studies on feather regeneration and molt done on spotted munia *L. punctulata* suggest that $T_4$ (mono-deiodination suppressed by iopanoic acid and thyroidectomized birds) is more effective than $T_3$ in inducing feather regeneration. The binding pattern of $^{125}$I labeled $T_3$ and $T_4$ has been investigated in the nuclei prepared from skin and liver tissues (samples obtained during different months) of spotted munia using scatchard plot analysis. The results show that binding capacity ($B_{\text{max}}$ – pmole/80 µgm DNA) of $^{125}$I- $T_3$ to nuclei of skin was significantly higher in November as compared to April and June, whereas the binding affinity ($K_d$-$10^{-9}$M$^{-1}$) was significantly lower in November as compared to April and June. During November, $B_{\text{max}}$ for binding of $T_3$ and $T_4$ did not vary in liver and skin nuclei but $K_d$ varied significantly. Binding capacity of $^{125}$I- $T_4$ to skin and liver did not vary but binding affinity of $^{125}$I- $T_4$ to skin was approximately 7 times higher than that of liver. The results suggest that $T_4$ does show a variation in binding pattern that co-relates to the molting pattern of spotted munia. These variations might play important role in different physiological phenomenon in this tropical bird. The experiments do point towards the possibility of independent role of $T_4$ as a hormone, however, further experiments need to be done to ascertain the role of $T_4$ in this model and work out the exact molecular mechanism of action.

**Keywords**: Molt, Scatchard plot, Spotted munia, $T_4$ receptor, Thyroid hormones, Thyroxin

Breeding is the key to survival of any species and it occurs when the environmental conditions are optimum for the upbringing of the young ones. In order to reproduce at the right time of the year, seasonal breeders such as birds must begin physiological preparations much earlier than actual reproductive events. The phenomenon of seasonality thus involves not only the adjustment of physiological events like breeding, molting, migration etc. with the external environment but also their mutual timing so as to preclude simultaneous occurrence of these energy requiring events. While considerable information is available (chiefly on temperate zone birds) on the environmental control of some of these events e.g. reproduction, migration and molt (relatively little), almost nothing is understood about the physiological strategies used for mutually spacing these events along the annual calendar.

Reproductive cycles of some Indian birds and their regulation have been investigated extensively but almost no long-term studies are available on seasonal molt and its control. Birds molt both to replace their worn out feathers and to change their appearance in either social signaling or predator avoidance context. Molting has high-energy demands and studies have shown that birds raise their basal metabolism by 9-46% while molt$^{1,3}$. Intense molt is thus at par with reproduction and migration, which also have high-energy demands$^{4,5}$. All these high energy demanding seasonal events, viz. molt, reproduction, migration etc. are known to be influenced by thyroid hormones. But almost nothing is understood as to how temporal spacing of high-energy seasonal events like molt, migration and reproduction is achieved in physiological terms. Also, almost no studies have been focused on measuring and quantifying the
binding patterns of T₄ and T₃ to the actual tissues involved in this physiological process.

Prevalent notion about thyroid hormones is that thyroxine (T₄) is a mere precursor and physiological effects of thyroid hormones are elicited by tri-iodothyronine (T₃). The major pathway for the production of T₃ is via 5′-deiodination of the outer ring of T₄ by deiodinases and accounts for the majority of the circulating T₃. Type I deiodinase is found in peripheral tissues such as liver and kidney and is responsible for the conversion of the majority of T₄ to T₃ in circulation. Type II deiodinase is found in brain, pituitary, and brown adipose tissue and primarily converts T₄ to T₃ for intracellular use. These deiodinases have been cloned and demonstrated to be selenoproteins.

Earlier studies conducted on feather regeneration and molt in spotted munia *Lonchura punctulata* suggest that T₄ (mono-deiodination suppressed by iopanoic acid and thyroidectomized birds) is more effective than T₃ in inducing feather regeneration. These studies also indicate that T₄ and T₃ might have independent hormone roles. In the present study, an attempt has been made to investigate the binding pattern of labeled T₄ and T₃ in the nuclei prepared from skin and liver tissues (samples obtained during different months) of spotted munia using scatchard plot analysis and correlated it to the strategy of temporal spacing of molt with reproduction.

**Materials and Methods**

*Study model*—Spotted munia (*Lonchura punctulata*), a tropical finch distributed all over the Indian sub-continent (08° 04′N to 37° 60′N to 97° 25′ E) was the study model. The breeding of this species coincides with the monsoon period. In the Indian sub-continent monsoon generally starts from June first week and prevails upto middle/last of September. In nature as well as in captivity the gonads of spotted munia begin to develop from May/June, attain peak of activity by September/October and regression generally occurs in November. However, birds were found to go through an entire cycle in captivity in a year (1979) which did not have a monsoon season. Therefore monsoon or humidity as such does not seem to serve as a proximate factor for reproduction in this species. Extensive studies on the regulation of breeding in spotted munia indicate that annual changes in daylength is the main environmental factor that synchronise an endogenous circannual cycle of breeding in this species with the period of abundant food supply (monsoon season). All the animal handling was as per ethical standards. Only four birds were kept in each cage during experimentation. Cages were cleaned everyday with water including food and water containers. Proper perching facility, clean water and food were available all the time. Care was taken to ensure that leg rings would not become too tight and cause injury. Whenever necessary, veterinary care was provided. Before and after experiments birds were set free in the semi natural aviary.

*Molting*—Birds were obtained and housed in the outdoor aviary. After 15 days of acclimatization, male and female birds were separated by laparotomy under ether anesthesia. Only male birds were used in the study. Birds were weighed and housed in wired cages (18x12x12 inches) and provided same diet throughout i.e. pearl millet and water available *ad libitum*. Grit and crusted egg-shells were given occasionally to all birds. Cages were housed in an aviary in ambient Natural Day Length (NDL) condition. A separate group was also maintained in artificial condition of continuous illumination (LL–150 lux, 25±3 ºC). Spotted munia and some other avian species has been shown to exhibit circannual rhythms under different light intensities (~22 lx -300 lux). In both the groups, wing molt was observed monthly according to Newton. This method essentially comprises an arbitrary scoring of 0 (old) and 5 (new, fully grown feather). For quantifying head molt, and arbitrary scale of 1 to 6 for feather loss in head and 1 to 6 for feather re-generation of head feather was devised. For this, head of the bird was divided into 6 parts and molt was observed in each part. Body molt and tail molt were also observed regularly each month. To record the gonadal development long and short axis of the left testis was measured *in situ* under ether anesthesia each month in all the birds. The molting events and gonadal development were correlated with the profile of thyroxin (T₄) and tri-iodothyronine (T₃) in NDL at the same latitude (30° 13′N) already published.

*Simulation of annual photocycle in six months*—(T6M)—Birds housed in the outdoor aviary were subjected to simulated photoperiodic cycle–T6. After 15 days of acclimatization, male and female birds were separated by laparotomy under ether anesthesia. Only male birds were used in the study. Birds were weighed and housed in wired cages (18x12x12 inches) and provided same diet throughout i.e. pearl millet and water available *ad libitum*. The breeding of this species with the period of abundant changes in daylength is the main environmental factor indicating that annual

THAPLIYAL et al.: BINDING PATTERN OF ¹²⁵I-T₄ AND T₃ IN TISSUES OF SPOTTED MUNIA

479
Grit and crusted egg-shells were given occasionally to all birds. Cages were housed in an avairy in ambient chambers with minimum interference from other birds. Annual changes in day length were compressed into 6 months and so the rate of day-length change was twice that of normal. All the parameters were measured under this schedule.

Seasonal peripheral conversion of T₄ to T₃—This study was performed four times a year – twice when the gonads were quiescent (February–April) and twice when the gonads were developing (August–September). For each set of study, 10 individuals were surgically thyroidectomized and kept in ambient laboratory conditions. Out of these, four were bled, 3 days later and plasma T₃ and T₄ were assayed to check for completeness of thyroidectomy. Four days following surgery, birds were injected with L-T₄ (Sigma–2.5 μg/0.1 mL saline) and bled 4 hours after the injection. Injections were administered at a fixed time in the morning during all four assays. Total plasma T₄ and T₃ concentrations were measured using in-house RIA¹⁰,¹⁹,²⁰. High specific activity ¹²⁵I T₃ (3000 μci/µg) and ¹²⁵I T₄ (2574 μci/µg) were obtained from BRIT Mumbai. Specific antisera (A/S Tony Meritt, U.K.) for T₄ and T₃ were a gift from Prof. Roger Ekins (Department of Molecular Endocrinology, Middlesex Hospital Medical School, London). T₄ A/S was used at 1:160,000 and T₃ A/S at 1:80,000 dilutions. Standards were prepared in avian hormone free serum. Methyl cellulose/charcoal suspension was used for the separation of free and bound hormone fractions. Quality controls were run with the samples in each assay for the assessment of specific binding and maximize specific binding by examining the effect of varying conditions like pH etc. Incubations were carried out at different pH (7.2, 7.4, 7.6, 7.8, 8.0), temperature (4 °C overnight, room temperature) and durations (2, 4, 6, 10, 12 h).

Effect of reverse Tri-iodothyronine (rT3)—This experiment was conducted to investigate the effects of rT₃, if any, and rule out the possibility that any of the T₄ effects may be due to its conversion to rT₃. Adult males and females were divided into four groups of six birds each (Group I to IV). Birds were deplumed on the right feather tract same day and hormone treatment was started. Group I, II and III received 0.37, 0.74 and 1.48 nM L-rT₃/day/bird (sodium salt, Sigma Chemicals: St Louis, MO) respectively in alkaline normal saline for 15 days. Group IV was given alkaline saline and served as control. The right feather tract was examined daily and the total number of feather papillae per unit area was noted. Feather count was obtained by placing a thin wire square (1 cm x 1 cm) along the center of the feather tract of the birds at three random sites and counting the numbers within (three times/unit area). Readings were started 24 hours after the last injection. Long and short axes of the left testis were measured in situ by laparotomy under ether anesthesia.

Preparation of purified nuclei—The standardization of technique used for preparation of nuclei was based on the method of Lawson et al²³. In short, liver and tissue from feather tract were placed in ice cold SMNaT (0.25 M Sucrose, 10 mM MgCl₂, 50 mM NaHSO₃, 10 mM Tris buffer, pH 7.8). The tissue was homogenized in SMNaT buffer in ice-cold Teflon-glass Potter-Elvehjem type homogenizer. Nuclear pellet was obtained by centrifugation (Sorvall, RC5C) at 15000 g at 0 °C for 20 min. After 3 washes with SMNaT buffer, protein/DNA content was measured (at 260 nm) spectrophotometrically (Beckman DU 640). The preparation was checked for debris through phase contrast microscope (Zeiss) with DAPI stain. Best nuclear preparations were used for the binding studies.

Optimization of binding assay—Optimization of binding assay was carried out to minimize non-specific binding and maximize specific binding by incubating the effect of varying conditions like pH etc. Incubations were carried out at different pH (7.2, 7.4, 7.6, 7.8, 8.0), temperature (4 °C overnight, room temperature) and durations (2, 4, 6, 10, 12 h).

Nuclear receptor binding assay for T₃ and T₄—For binding studies, isolated and purified nuclei from liver and feather tract skin (≥ 80 μg DNA/tube) were suspended in SMCT buffer (0.32 M Sucrose, 3 mM MgCl₂, 2 mM CaCl₂, 2.5 mM DTT, 10 mM Tris buffer, pH 7.8) with increasing amount of ¹²⁵I T₃

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Table 1—Characteristics of in-house T₄, T₃ RIA’s (Pant and Chandola -1993)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>T₄</th>
<th>T₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.91</td>
<td>0.957</td>
</tr>
<tr>
<td>Slope</td>
<td>0.0059</td>
<td>0.003</td>
</tr>
<tr>
<td>Precision index</td>
<td>0.0543</td>
<td>0.033</td>
</tr>
<tr>
<td>Percent coefficient variation</td>
<td>0.41 – 5.9</td>
<td>2.5 – 9.3</td>
</tr>
<tr>
<td>Inter assay variation</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>
started from January and lasted almost for six months. Wing molt shedding started in September/October and regeneration was completed by December. In birds kept under NDL schedule, pattern of body, head, and wing molt process (head, body and wings) was hastened; the wing molt was initiated during January and competed by May. The head molt started in August and the regeneration was completed by November. The gonads started developing early i.e. April/May (May, NDL vs LL, P < 0.01) and attained peak by July and started to regress thereafter (Fig. 1b).

Simulation of annual photocycle in six months – (T6 M)—Under simulated annual photo cycle of six months periodicity testicular volume, T3, T4 and molting cycles were almost compressed into six months and nearly maintained their spatial and temporal spacing as observed in NDL (Fig. 1c). Testicular volume along with T3 profile peaked during simulated September/October (calendar month – May). For the second cycle, testicular volumes started increasing during simulated October and peaked by simulated December (calendar month – December). T4 levels, measured for the first simulated cycle only, were at baseline till simulated October and peaked during simulated December/January. Molting was initiated in simulated January and ended by simulated July/August. Head and body molt was occurred between simulated October/November to simulated February/March.

Seasonal peripheral conversion of T4 to T3—Peripheral conversion data show that percent T4 to T3 conversion is low during off breeding phase (February and April - 0.020 and 0.024% respectively) and high during the breeding period (August and September - 0.60 and 0.75% respectively) and this difference was significant (February vs August/September, P<0.001), April vs August/September, P < 0.001) (Fig. 2).

Effect of rT3 and T4—There was no significant difference in number of regenerating feather papillae per unit area in birds of groups I, II and III as compared to group IV (control) on the 5 th day (0.37, 0.74, 1.48 nM rT3 vs saline, P = ns for all doses). There was a significant increase in number of feather papillae/cm² in groups receiving 0.74 nM T4 as compared to saline control (P<0.05). Birds treated with 0.37, 0.74, 1.48 nM rT3 did not show any significant decrease in testicular volume as compared to the control group (0.37, 0.74, 1.48 nM rT3 vs saline, P = ns for all doses).

Results

Molting—Observation on molting shows distinct pattern of body, head, and wing molt. Body and head molt occurred in two distinct phases of shedding and regeneration. In birds kept under NDL schedule, shedding started in September/October and regeneration was completed by December. Wing molt started from January and lasted almost for six months till June/July. The gonads started developing from May/June and attained peak in September/October and regressed completely by December (Fig. 1a). The birds kept under LL show a shift in molt pattern and gonadal cycle. Molting process (head, body and wings) was hastened; the wing molt was initiated during January and competed by May. The head molt started in August and the regeneration was completed by November. The gonads started developing early i.e. April/May (May, NDL vs LL, P < 0.01) and attained peak by July and started to regress thereafter (Fig. 1b).
Optimization of binding assay—Optimizations were carried out to minimize non-specific binding and maximize specific binding by examining the effect of varying condition like pH, temperature and time. Maximal binding of T₃ and T₄, with reproducible results were obtained at pH 7.8, room temperature and after 4 h of incubation. Non-specific binding was high for T₄ (approx. 20-25%) and less for T₃ (10-15%).

Characteristics of T₄ and T₃ nuclear binding—Nuclear preparation from both the tissues (liver and
skin) showed specific saturable binding sites for both T₃ and T₄ with limited capacity and high affinity receptors. Binding characteristics of ¹²⁵I-T₃ to liver and feather tract are summarized in Tables 2 and 3. During April and June, binding capacity (Bₘₐₓ) and binding affinity (Kd) of ¹²⁵I-T₃ for liver showed no significant variation (Bₘₐₓ and Kd, all values compared, P=ns). During November, binding capacity of ¹²⁵I-T₃ to skin was significantly higher as compared to April and June (Bₘₐₓ, April vs November, P<0.001 and June vs November, P<0.001) whereas during same period, binding affinity was significantly lower (Kd, April vs November, P<0.01 and June vs November, P<0.01).

During November, binding capacity of ¹²⁵I-T₃ and ¹²⁵I-T₄ to liver and skin did not vary significantly (Bₘₐₓ, liver vs skin, P=ns) but binding affinity varied. Binding affinity of ¹²⁵I-T₃ to liver and skin did not vary (Kd, liver vs skin, P=ns). Binding affinity of ¹²⁵I-T₄ to skin was approximately 7 times higher than that of liver (Kd, liver vs skin, P<0.001). Binding affinity of ¹²⁵I-T₃ to liver was approximately double as compared to that of ¹²⁵I-T₄ (Kd, liver, ¹²⁵I-T₃ vs ¹²⁵I-T₄, P<0.05). Binding affinity of ¹²⁵I-T₄ to feather tract skin in November (during molt) was approximately 3 ½ times more than that of ¹²⁵I-T₃ (Kd skin, November, ¹²⁵I-T₃ vs ¹²⁵I-T₄, P<0.001) (Figs. 3 and 4; Table 2).

Competitive binding studies—Nuclear binding of ¹²⁵I-T₃ decreased significantly by saturating amounts of T₃. 50% inhibition resulted from 100 fold increase. Saturable amounts of T₄, on the other hand, did not inhibit nuclear binding of ¹²⁵I-T₃ to comparable levels even by 100 or 1000 fold increase (Fig. 5).

**Table 2**—Binding of ¹²⁵I-T₃ to nuclei purified from liver and feather tract skin tissue (Bₘₐₓ - p moles/80 µgm DNA, Kd - 10⁻⁹ M⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>April (non-molting period)</th>
<th>June (None molting(Onset of body period-gonads molt) growing)</th>
<th>November (Onset of molt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₃</td>
<td>Liver Bₘₐₓ</td>
<td>178.12 ± 05.06</td>
<td>155.01 ± 8.49</td>
</tr>
<tr>
<td></td>
<td>Kd</td>
<td>0.398 ± 0.037</td>
<td>0.220 ± 0.060</td>
</tr>
<tr>
<td>Feather Bₘₐₓ</td>
<td>175.70 ± 14.32</td>
<td>148.52 ± 16.21</td>
<td>280.42 ± 17.64</td>
</tr>
<tr>
<td></td>
<td>Kd</td>
<td>0.177 ± 0.085</td>
<td>0.114 ± 0.016</td>
</tr>
</tbody>
</table>

**Table 3**—Binding of ¹²⁵I-T₄ to nuclei purified from liver and feather tract skin tissue during onset of molt Bₘₐₓ - p moles/80 µgm DNA, Kd - 10⁻⁹ M⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>November (Onset of body molt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₄</td>
<td>Liver Bₘₐₓ</td>
</tr>
<tr>
<td></td>
<td>Kd</td>
</tr>
<tr>
<td>Feather Bₘₐₓ</td>
<td>149.43 ± 8.69</td>
</tr>
<tr>
<td></td>
<td>Kd</td>
</tr>
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</table>

**Fig. 2**—Seasonal variations in extrathyroidal conversion of T₄ and T₃ and T₃/T₄ ratio (February, April Vs August, September ***P<0.001)

**Fig. 3**—*In vitro* binding of ¹²⁵I-T₃ to purified nuclei from liver (a) and skin (b) of spotted munia (November). [R² varied from 0.9 – 0.95 and six assays were performed for each sample].
Fig. 4—*In vitro* binding of $^{125}$IT$_4$ to purified nuclei from liver (a) and skin (b) of spotted munia (November). [R$^2$ varied from 0.8 – 0.9 and six assays were performed for each sample].

Fig. 5—Competitive inhibition of binding of $^{125}$IT$_3$ to nuclear preparation from skin tissue by cold T$_3$ and T$_4$.

**Discussion**

**Molt**—In spotted munia under NDL conditions, when pattern of molt is correlated with plasma profiles of T$_4$ and T$_3$, T$_4$ concentration seems to be associated with the process of molting. Head molting starts as soon as plasma concentration of T$_4$ begins to rise (September/October) and the process is completed when T$_4$ levels reach peak (December) triggering wing molt. During molt, T$_3$ levels, however, remain minimal throughout. When wing molt is completed (June/July) and gonadal growth is initiated, T$_4$ levels reach minimal values and remain low throughout the reproductive period. T$_3$, on the other hand, increases. Recent studies also suggest that hormonal profiles of T$_4$ rather than T$_3$ correlate with molting.

Results also indicate that in LL condition, the molt and gonad cycles showed advance shift. Molting commences in January and completes in May. Gonads start to develop in May and attain peak in July. Head molt commences in August and is completed by November. From this, it may be assumed that in LL, T$_4$ levels start rising in August and attain peak by November (as head molt completes) and decline earlier as compared to NDL so that the gonads could develop. Thus, although the physiological events shifted by about two months in LL, the mutual phase relationship was nearly maintained.

Besides spotted munia, in ducks and teals, house sparrow, domestic turkeys, Canada goose, lesser snow goose, and Emperor penguins annual maximum plasma T$_4$ levels were found to be coincident with the period of molt. Inter-relationship of testosterone and thyroid has also been implicated in the physiology of molt in European starling.

Results of peripheral conversion of T$_4$ to T$_3$ showed marked seasonal variation, low during molting and high during breeding/non-molting period. Evidently, high concentration of T$_4$ in molting birds and low T$_4$ in breeding birds are a consequence of seasonal variation in peripheral conversion of T$_4$ to T$_3$. Therefore, it may be suggested that in spotted munia a seasonal variation in peripheral mono-de-iodination of T$_4$ in accordance with the demands of the seasons may determine the expression or inhibition of particular thyroid-dependent seasonal event such as breeding and molting. When the rate of peripheral conversion is high, more T$_4$ is converted to T$_3$, eventually lowering the T$_4$ level and low T$_4$ concentration is conducive to gonadal development. As the rate of peripheral conversion goes down, less of T$_4$ is mono-de-iodinated and hence T$_4$ levels start increasing. This increase of T$_4$ level appears to initiate the chronological process of molting. After peak levels of T$_4$ are attained, the wing molt is initiated. After initiating wing molt, a minimum basal level of T$_4$ appears to be enough to continue the process. Thus it seems that mono-de-iodination of thyroxine may be used as a subtle control to achieve temporal spacing of molt and reproductive cycle in spotted munia.
High T₃ levels during breeding period at first glance might suggest a gonado-stimulatory role for T₃ but since removal of thyroid gland accelerates gonadal development and fat deposition and therefore the gonado-stimulatory role of T₃ is ruled out. Thus increasing/high concentration of T₄ ensures initiation of molting while keeping the gonads suppressed. Further, in vivo studies done on spotted munia have already established that some effects, like gonadal regression may be brought about directly by T₄ alone, without prior conversion of T₃. On the other hand, the effect of feather regeneration may involve action of both T₄ and T₃ independently.²⁴

It is known that almost all of the T₃ present in plasma is derived from the extra-thyroidal conversion of T₄ to T₃.²⁵⁻²⁷ Environmental factors like food availability and temperature are also known to significantly affect peripheral conversion of T₄ to T₃.²⁰⁻²⁴ It is interesting to note that the plasma T₄ cycle in spotted munia runs almost parallel to the food consumption cycle. Further, athyroidic birds continue to show seasonal variations in food consumption and restriction of food intake lowers thyroid activity.²⁸ Thus it seems that annual changes in food consumption may constitute one of the major factors contributing to seasonal thyroid cycle in spotted munia.

Temperature is well known to affect thyroid functions.¹¹⁻¹³ Like other birds, in spotted munia also, thyroid activity is high in winter and low in summer. However, in birds maintained in LL, molt cycle (and hence thyroid cycles) showed a shift despite almost constant temperature. Under artificially shortened photocycle of six months also these cycles were compressed to about six months periodicity. Subsequently, T₄ and T₃ levels in T6 birds were also found to be phase shifted in accordance with the seasonal molt and reproduction. This was remarkable because in this experiment only the photo-cycle was compressed and no alterations in temperature were made. Obviously while fluctuations in temperature may influence thyroid activity, photoperiod appears to be the riding force.

Present result also points out towards differential tissue responses to endogenous T₄ levels. It seems that different tissue viz. head skin, body skin (feather tract) and skin of wings respond to different concentration of endogenous T₄ or in other words– have different threshold requirement. For example when the circulating T₃ concentration just start rising – the feathers of head commence molting; half-way through the rising – the body feathers molt and; the wing molt starts when the T₄ levels peak. It seems that high T₄ levels are required only to initiate the process of wing molt and are not essential thereafter because the molt progresses despite a decline in T₄ levels. Results with thyroid ablation show immediate arrest of molt (unpublished data) indicating that minimal circulating thyroid hormone is required for the process of molting to be completed.

Results with the rT₃ indicate that in both series of experiments, rT₃ did not have a stimulatory effect on feather regeneration. Earlier experiments related to feather regeneration, with replacement therapy by T₄ or T₃ in thyroidectomized birds suggest that T₄ can be more effective than T₃ in inducing feather regeneration.⁵⁴ Similarly, T₄ has been shown to be anti-gonadal in spotted munia but T₃ did not mimic this gonado-inhibitory effect. From the present results it is clear that the greater efficacy of T₄ to stimulate feather regeneration as compared to T₃ and the failure of T₃ to mimic the inhibitory effect on gonads cannot be attributed to the conversion of T₄ to rT₃.

The binding experiments show that liver tissue has approximately same binding characteristics for ¹²⁵I-T₃ during the study but binding characteristics of ¹²⁵I-T₃ to skin varies. Results indicate that that during molting period both T₄ and T₃ (¹²⁵I) has approximately same binding capacity to liver and feather tract skin nuclei but their binding affinities varies. During this period the binding affinity of T₄ to feather tract skin nuclei is approximately 7 times higher than that of liver and 3½ times more than that of T₃ to feather track skin. It is the time when molting in head and body feathers is induced and T₄ concentrations start rising. High affinity of T₄ to skin may probably sensitize the skin to very minute rise in T₄ concentration and may ultimately lead to induction of molting. The present data also show that binding affinity and capacity of T₃ to liver tissue does not vary significantly. This is understandable as liver is an organ which is central to metabolism and thyroid hormones effect on its tissue should be at optimum level, most of the time. Studies have been performed by Oppenheimer et al.⁵⁵ on nuclear binding of T₃ with various tissue of rat like liver, kidney, pituitary, heart, brain, spleen and testis. Data from their studies demonstrated that concentration of binding sites for T₃ in tissues varied.

A high degree of specificity for T₄ as well as T₃ was also demonstrated by saturation analysis.
Competitive inhibition studies in spotted munia showed that nuclear binding to skin nuclei to $^{125}$I$\text{T}_3$ was decreased significantly by saturating amounts of $\text{T}_3$. 50% inhibition resulted from a 100 fold increase. Saturable amount of $\text{T}_4$, on the other hand, did not inhibit nuclear binding of $^{125}$I$\text{T}_3$ to comparable levels even by 100 or 1000 fold increase (Fig. 5). This demonstrates two facts: first - $\text{T}_4$ and $\text{T}_3$ do not bind to same sites and second - sites that bind $\text{T}_3$ have poor affinity for $\text{T}_4$. These results, although preliminary, also demonstrate that $\text{T}_4$ has binding sites in skin of feather tract which is a potential target tissue for thyroid hormones. This also argues for possible different binding sites of $\text{T}_4$ and $\text{T}_3$. In hepatic nuclei of pre-metamorphic tadpoles the affinity of $\text{T}_3$ was twice than that of $\text{T}_4$.

Moreover, in the present study, the binding pattern to this tissue specificity $\text{in vitro}$ has also been correlated in vivo 

The present study, cellular/tissue specificity has also been demonstrated which enables target tissue (in this case liver and skin) to respond to specific hormones present in circulation. Of special interest is that the present $\text{in vitro}$ investigations have demonstrated the presence of high affinity and limited capacity binding sites for $\text{T}_4$ in skin. This is one of the first reports, to the best of our knowledge, identifying thyroid hormone receptors/binding proteins in skin. Previous $\text{in vivo}$ studies on spotted munia had shown that $\text{T}_3$ need not necessarily mimic $\text{T}_4$ effects and $\text{T}_3$ evoked greater feather growth and regeneration. These studies identified skin as a potential target organ for $\text{T}_4$ to act as hormone. Moreover, in the present study, the binding pattern can be correlated to a biological response which is an important criterion for receptor characterization. In the present context although a tissue/cellular specific binding site has been demonstrated in target organ $\text{in vitro}$, biological response has also been correlated to this tissue specificity $\text{in vivo}$. Demonstration of $\text{in vitro}$ receptor-dependent hormonal response mechanisms are yet to be achieved in the present case. Thus it can be concluded that the present results point out towards a distinct possibility of independent and distinct role of $\text{T}_4$ as hormone acting via its own receptors and helping in timing/synchronization of seasonal cycles of breeding and molt in spotted munia.

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