Flight enhances juvenile hormone inactivation in *Danaus plexippus plexippus* L. (Lepidoptera: Danaidae)

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Summary. Adult Monarch butterflies injected with ^{3}H-juvenile hormone (JH) showed a significant increase in haemolymph JH metabolites after a 40 min flight compared to unflown controls. In addition, haemolymph enzymatic JH metabolism was shown to increase with thoracic temperature increases previously shown to be associated with flight.

In the Fall of each year the North American Monarch butterfly, *Danaus plexippus plexippus* L., undertakes long migratory flights to overwintering sites, and during this migration reproductive tract development is substantially reduced in both sexes[2]. The reduced development of the JH-sensitive Monarch reproductive organs during migration suggests that flight behaviour might somehow reduce effective JH titers at the reproductive tract and thereby inhibit inappropriately timed reproduction. Although haemolymph JH titers of Monarchs during the actual act of migration are not available, migrant JH titers measured at various time intervals after field capture and laboratory confinement are perplexingly variable[3]. This result suggests that the JH titer increases after the absence of migratory flight behaviour. We have therefore examined the effect of flight on JH inactivation in Monarch butterflies.

In our 1st series of experiments, we examined the metabolism of JH in flown and unflown Monarch males and females. Most of the experimental animals were left intact, but some were ligatured between the thorax and abdomen prior to removal of the abdomen. These latter butterflies (i.e., the head-thorax preparations) were used to eliminate possible complications arising from JH uptake by abdominal target tissues. Intact animals and head-thorax preparations, respectively, were injected with 5×10^4 cpm (1400 pg) or 2×10^4 cpm (560 pg) ^{3}H-JH I (New England Nuclear) dissolved in 10 µl of 10% ethanol. They were immediately flown on a flight mill, or immobilized to prevent wing movement, for 40 min at room temperature (23–25 °C).

At the end of the flight period, haemolymph was withdrawn from the thorax with calibrated, carbowaxed (mol.wt 20,000, Sigma), micropipettes, and both intact ^{3}H-JH I and ^{3}H-JH I metabolites were quantified by the isooctane: methanol partition method[4]. The results of these experiments (table 1) showed no significant differences in total haemolymph radioactivity in either group, and no differences of any kind between males and females. However, the data (table 1) clearly demonstrated a 50–82% increase in ^{3}H-JH I metabolites in flown intact and head-thorax butterflies. Although both flown and unflown animals metabolized ^{3}H-JH I, the flown animals did so at an augmented rate. Expressed another way, the data (table 1) may be converted to the average percent haemolymph ^{3}H-JH I metabolized. In this form 47.3% (flown) versus 41.7% (unflown) of haemolymph cpm were due to ^{3}H-JH I metabolites in intact animals, and 56.9% (flown) versus 40.0% (unflown) of haemolymph cpm were due to ^{3}H-JH I metabolites in head-thorax preparations. It would be expected that flight times progressively longer than 40 min would yield increasingly greater differences between flown and unflown animals.

We next examined the possibility that the results of the above experiments might have been due to changes in haemolymph volume, or differential uptake of labeled material by internal tissues, during Monarch flight. The former possibility was discounted when injection of 1×10^5 cpm of ^{3}H-inulin (New England Nuclear) into intact animals prior to a flight experiment indicated haemolymph volume of 79 ± 5 µl and 67 ± 8 µl (mean ± SEM), respectively after a 40 min experiment using 10 flown and 10 unflown intact Monarchs. Similarly, analysis of chloroform:methanol extracts[5] of all internal tissues at the end of a flight experiment demonstrated no apparent variation of intact ^{3}H-JH I, and equal amounts (about 25% of total tissue radioactivity) of labeled ^{3}H-JH I metabolites, in the internal tissues of flown and unflown intact animals.

Subsequent studies examined the possible mechanism by which Monarch flight might act to enhance JH inactivation. These experiments were directed by the recent demonstration of a JH-specific esterase (JHE) in haemolymph from both Monarch sexes[3,6], and by an earlier report of a 6–10 °C increase in thoracic temperature during Monarch flight[7]. Using in vitro methodology we first showed that JHE activity in whole Monarch haemolymph, and the activity of partially purified JHE obtained by Sephadex G-100 gel filtration of Monarch haemolymph[3,6], was significantly elevated when the incubation temperature was raised from 25 to 35 °C (table 2). Furthermore, in vivo experiments in which head-thorax preparations were preincubated at 25 or 35 °C for 30 min, injected with 5×10^4 cpm ^{3}H-JH I, and then returned to their respective temperatures for an additional 40 min, clearly showed enhanced inactivation of ^{3}H-JH I at the higher temperature (table 2). Since all animals in these latter studies were held immobile in identical fashion, these results also argued against some unknown mechanical factor during flight (e.g., increased heart rate) being responsible for our initial observations.

To examine further the possible biological significance of temperature effects on JHE activity, reproductively inactive female Monarchs were neck-ligurated to remove cephalic endocrine influences[8,9], and then injected with 10 µg JH I

Table 1. Effects of flight on production of juvenile hormone metabolites in Monarch butterflies

	cpm/5 µl haemolymph	
	Flown	Unflown
Intact	(17)	(16)
Total	2550 ± 331	1927 ± 163
JH metabolites	*1207 ± 177	803 ± 86
Head-thorax only	(9)	(10)
Total	2193 ± 331	1717 ± 283
JH metabolites	*1247 ± 234	685 ± 111

Data presented as $\bar{X} \pm$ SEM; ( ), number of animals; *significantly different from unflown at $p < 0.05$ in Student's t-test; all animals were injected with ^{3}H-JH I and held at 23–25 °C for 40 min; haemolymph samples were examined for JH metabolism by isooctane: methanol partition[4]. 5 µl of haemolymph was blown into a test tube containing 295 µl 5 mM tris-HCl buffer (pH 7.4) with 0.1 M NaCl and 60 mg/l glutathione, 750 µl isooctane, 75 µl methanol, 67.5 µl H_2O and 7.5 µl concentrated ammonium hydroxide. Each tube was vortexed 6 times (5 sec/time) on a scientific products vortex mixer. A partition was obtained after centrifugation at 10,000 rpm for 15 min. The phases were removed to separate scintillation vials using 500 µl fixed needle Hamilton syringes. After addition of 10 ml Aquasol II to each vial, radioactivity was quantified using a Beckman LS-100C scintillation counter. Quenching was determined by both internal and external standard methodology. The data reflect a correction for the slight quench observed in the aqueous phase.

(Calbiochem) in 10 µl mineral oil (U.S.P.). Injected animals were held at 25 or 35 °C (with a 16 h photophase) and injected daily with 10% sterile glucose to maintain life. On the 6th day females were dissected and the condition of their JH-sensitive reproductive organs[8,9] evaluated. The results demonstrated significantly greater responses to JH at 25 °C; ovarian and colleterial gland wet weights were 50±4 mg and 8±1 mg, respectively, in 9 females held at 25 °C, while the respective values were 25±5 mg and 3±1 mg in the 8 females held at the higher temperature.

Thus, we have shown that both intact Monarchs and those lacking abdomens exhibit significantly elevated JH I inactivation during flight, and the enhanced metabolism of JH does not appear to be due to alterations in haemolymph volume or tissue uptake of JH while flying, or to mechanical factors associated with flight. In addition, evidence presented here and elsewhere[3,6] demonstrates the existence of a JHE in Monarch haemolymph, and our in vivo and in vitro experiments indicate, not surprisingly, that temperature can influence JH metabolism by JHE. These observations, coupled with the known elevation of thoracic temperature during Monarch flight[7], suggest the hypothesis that flight-induced elevations of thoracic temperature may act to increase haemolymph JHE activity and thereby reduce haemolymph JH titers in flying Monarchs. Since a large number of insects use JH to regulate adult reproductive tract development[10], and many insects also exhibit elevated thoracic temperatures during flight[11-13], it may be that this phenomenon occurs in many insects exhibiting adult flight behaviour. Behaviour control of gonadotropins, although apparently a novel concept with respect to insects, has been demonstrated in other animals[14].

Table 2. Influence of incubation temperature on in vivo and in vitro hydrolysis of ^{3}H-JH I by adult Monarch haemolymph JHE

	% ^{3}H-JH I hydrolyzed	
	25 °C	35 °C
In vitro		
Whole haemolymph (14)	75±3	*85±1
JHE fractions (5)	46±3	*61±5
Buffer alone (8)	14±5	16±3
	cpm/5 µl haemolymph	
	25 °C	35 °C
In vivo		
Total (10)	3688±207	4379±359
JH metabolites (10)	2012±110	*3179±278

Data are expressed as $\bar{X}\pm$SEM; ( ), number of animals of column fraction aliquots tested per point; * significantly different from 25 °C control at $p<0.05$ using the Student's t-test. Assay conditions in vitro: 1 µl whole haemolymph was added to 340 nM ^{3}H-JH I in 100 µl of 5 mM tris-HCl buffer (pH 7.4) with 0.1 M NaCl and 60 mg/l glutathione; incubated 15 min then a partition was formed and ^{3}H-JH I metabolites quantified as described in the table 1 legend. JHE fraction aliquots (100 µl) were obtained by chromatographing 100 µl of haemolymph on a Sephadex G-100 column (1.5×65 cm) equilibrated in the above buffer. 2 ml fractions at flow rate of 30 ml/h were collected; 100 µl aliquots of each fraction were tested for JHE activity by incubation with 340 nM ^{3}H-JH I followed by isooctane:methanol partition. The fractions of the JHE activity peak were pooled and 100 µl aliquots were tested for JHE activity at 25 and 35 °C as described above for whole haemolymph. Assay conditions in vivo: head-thorax preparations were preincubated at either 25 or 35 °C for 30 min, then injected with 5×10^4 cpm (1.4 ng) ^{3}H-JH I and incubated for an additional 40 min at their respective temperatures. Haemolymph (5 µl) was removed from the thorax of each animal and the amounts of JH and JH metabolites were determined by isooctane:aqueous methanol partition as described in the table 1 legend.

High temperature inactivation of JH is not a new concept. It was noted 20 years ago that JH active *Hyalophora cecropia* extracts had differential effects on *Antherea polyphemus* pupae incubated at 9, 25, and 35 °C[15]. At 35 °C the extracts had little effect and this was assumed to be due to inactivation. In addition, the temperature optima for larval *Manduca sexta* JHE activity have been determined to be 30 °C[16] and 50 °C[17], indicating that temperature influences JH degradation in larval lepidopterans. However, since larvae do not fly, and thus are not endothermic, temperature-related JHE activity changes would presumably occur according to ambient environmental temperatures or direct absorption of solar radiation.

JH, which is biosynthesized in the corpora allata (CA), must pass through the thorax to reach the abdominal target tissues. The adult insect thorax is the only body region which increases significantly in temperature during flight[11,12]. In fact, the adult insect head, containing the JH biosynthesizing CA, remains at or below ambient during flight by evaporation of regurgitated fluid[18]. We believe, therefore, that the adult insect thorax is a type of JH-gauntlet and that the amount of JH which passes through the thorax intact would depend upon the rate of JH biosynthesis, the JHE titer, and the thoracic temperature.

At present, since the methodology to monitor JH biosynthesis in vivo is lacking, we do not understand the cause and effect relationship between migratory flight and JH biosynthesis. Clearly, neuroendocrine influences might simultaneously stimulate migratory flight and depress JH biosynthesis. However, one line of evidence argues against this hypothesis. JH has been implicated in controlling both flight muscle integrity and migratory flight behaviour[19]. Of particular relevance is the finding that in female *Oncopeltus* intermediate JH titers were associated with both migration and the absence of ovarian growth[20]. Ovarian growth occurred only in high JH titer groups, while both high and low JH titer groups lacked migratory flight behaviour. The latter appears analogous to that observed in overwintering, colonized Monarchs. Haemolymph JH titers are low or undetectable in colonized Monarchs found overwintering in California[3,6]. The mechanism controlling JH titer in overwintering Monarchs has not been fully elucidated, but probably involves a neuroendocrine component[21].

Assuming that JH is involved in Monarch migration, it is possible that flight-linked JH inactivation allows a significant JH titer to occur only locally in the region of the head and thoracic flight muscles, thereby stimulating migratory flight while inhibiting reproductive tract growth. Alternatively, we could assume that JH has little to do with Monarch migration. In this case, flight-linked JH inactivation may be used to destroy JH spuriously produced in response to neuroendocrine influences related to unusual, local environmental conditions encountered along the migration route. In either case, inappropriately timed JH-stimulated reproductive tract growth would be thwarted by flight-linked JH inactivation.

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2 F.A. Urquhart, The Monarch Butterfly. Toronto University Press, Toronto 1960.
3 C.A. Lessman, Ph.D. thesis, University of Minnesota, Minneapolis 1979.
4 B.D. Hammock and T.C. Sparks, Analyt. Biochem. *82*, 573 (1977).
5 J. Folch, M. Lees and G. Sloane-Stanley, J. biol. Chem. *226*, 497 (1957).
6 C.A. Lessman and W.S. Herman, Am. Zool. *18*, 342 (1978).

7 A.E. Kammer, Z. vergl. Physiol. *68*, 334 (1970).
8 J.F. Barker and W.S. Herman, J. exp. Zool. *183*, 1 (1973).
9 W.S. Herman, Gen. comp. Endocr. *26*, 534 (1975).
10 F. Engelman, The Physiology of Insect Reproduction. Pergamon Press, Oxford 1970.
11 B. Heinrich, Am. Scient. *65*, 455 (1977).
12 N.S. Church, J. exp. Biol. *37*, 186 (1960).
13 J.E. Heath and P.A. Adams, Nature *205*, 209 (1965).
14 C.G. Thibault, in: Oogenesis, p.397. Ed. J.D. Biggers and A.W. Schuetz. University Park Press, Baltimore 1972.
15 L.I. Gilbert and H. Schneiderman, Trans. Am. microsc. Soc. *79*, 38 (1960).
16 G.J. Weirich and J. Wren, Life Sci. *13*, 213 (1973).
17 R.K. Vince and L.I. Gilbert, Insect Biochem. *7*, 115 (1977).
18 B. Heinrich, Science *205*, 1269 (1979).
19 M.A. Rankin, in: Evolution of Insect Migration and Diapause, p.5. Ed. H. Dingle. Springer, New York 1978.
20 M.A. Rankin and L.M. Riddiford, J. Insect Physiol. *24*, 31 (1978).
21 W.S. Herman, J. Insect Physiol. *19*, 1883 (1973).

The effect of bile salts on thyroxine 5'-monodeiodination in rat liver homogenate[1]

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Summary. Unconjugated and conjugated bile salts inhibited the conversion of thyroxine to 3,3',5 triiodothyronine in rat liver homogenate.

The conversion of thyroxine(T_4) to 3,3',5 triiodothyronine (T_3) has been demonstrated to occur in the liver[2-4], kidney[5,6] and pituitary[7] of animals. Factors affecting this conversion are known to include fasting[4], thyroid state[8], sulfhydryl groups[7,9] and drugs[2]. However, little information is available about the significance of bile acids in this conversion. Our previous study in vivo[10] demonstrated that deoxycholate(DCA) feeding lowered the plasma T_3 levels in rats, and this was restored by additional cholestyramine feeding, suggesting that bile acid might affect the conversion of T_4 to T_3. In order to ascertain this assumption, the effect of bile salts such as sodium deoxycholate(SDCA), sodium cholate(SCA) and their tauro-conjugates on the in vitro conversion of T_4 to T_3 by rat liver homogenate was examined in this study.

Materials and methods. Sprague-Dawley male 70-day-old rats were fed a diet AO, which was prepared by removal of KI from the composition of diet A[11] in order to decrease iodine content to about 0.3 µg of iodine per g diet[12], for 7 days. These rats were decapitated to remove the liver. The liver was homogenized with 0.15 M phosphate buffer (pH 7.5) at 4 °C and the homogenate was incubated with a substrate, 50 µg T_4/ml, for 1 h at 37 °C, according to the method of Chopra[2].

SDCA and SCA were obtained from Nakarai Chemicals, Ltd. Sodium taurodeoxycholate(STDCA) and sodium taurocholate(STCA) were purchased from Sigma Chemical Co. Propylthiouracil (PTU) was donated by Tokyo Tanabe Co. Addition of these chemicals to the incubation medium resulted in no change of pH.

The amount of T_3 generated during incubation was measured by radioimmunoassay[13]. This assay system was not affected by any test substances in the concentrations used in this study.

Results. Both SDCA and SCA in concentrations between 10^{-1} M and 10^{-4} M significantly inhibited the conversion of T_4 to T_3 in rat liver homogenate. These effects were dose-related, but insignificant at a concentration of 10^{-6} M (table 1). In addition, SDCA was consistently less potent than SCA. The effect of PTU (2×10^{-5} M) in inhibiting T_4 to T_3 conversion was less than that of SCA at a concentration of 10^{-1} M ($p < 0.001$), but not significantly different from that of SDCA (10^{-1} M). The effects of conjugated bile salts on T_4 to T_3 conversion were also inhibitory (table 2), being significant at a concentration of 10^{-6} M. There was no significant difference between the effects of STDCA and STCA in all concentrations used.

Discussion. The present study demonstrates that both unconjugated and conjugated bile salts exert inhibitory effects on the hepatic conversion of T_4 to T_3 in the rat and that conjugated forms may be more potent than those of

Table 1. Effect of unconjugated bile salts and PTU on T_4 to T_3 conversion in rat liver homogenate

Bile salts	T_3 generated during incubation (ng/µg T_4/h/g-eq. tissue) SDCA	SCA	p-value (SDCA vs SCA)
10^{-1} M	9.82±0.27*	7.65±0.25*	<0.001
10^{-3} M	12.31±0.59*	10.50±0.45*	<0.05
10^{-4} M	15.50±0.25*	12.13±0.30*	<0.001
10^{-6} M	21.24±0.24**	19.23±0.70**	>0.05
PTU 2×10^{-5} M	9.41±0.25		
Control	20.33±0.30		

Values are expressed as the mean±SE in each group of 4 rats. Differences from control are shown by * $p<0.001$ and ** $p>0.05$.

Table 2. Effect of conjugated bile salts on T_4 to T_3 conversion in rat liver homogenate

Bile salts	T_3 generated during incubation (ng/µg T_4/h/g-eq. tissue) STDCA	STCA	p-value (STDCA vs STCA)
10^{-1} M	6.50±0.96*	5.50±0.65*	NS
10^{-4} M	12.00±0.71*	10.75±0.48*	NS
10^{-6} M	18.50±0.28**	18.30±0.24**	NS
Control	20.25±0.63		

Values are expressed as the mean±SE in each group of 4 rats. Differences from control are shown by * $p<0.001$ and ** $p<0.05$. NS indicates no significant difference ($p>0.05$).