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Stone, M. P., & Borer, P. N. (1981a) *Biophys. Chem. 14*, 363–368.

Stone, M. P., & Borer, P. N. (1981b) *Biophys. Chem. 14*, 369–374.

Stone, M. P., Johnson, D. L., & Borer, P. N. (1981) *Biochemistry 20*, 3604–3610.

Tiers, G. V. D., & Kowalsky (1960) *Abstracts of Papers*, 137th National Meeting of the American Chemical Society, Cleveland, OH, p 17R.

Ts'o, P. O. P. (1974) in *Basic Principles in Nucleic Acid Chemistry*, pp 454–584, Academic Press, New York.

Ts'o, P. O. P., Melvin, I. S., & Olsen, A. C. (1963) *J. Am. Chem. Soc. 85*, 1289.

Van Geet, A. L. (1968) *Anal. Chem. 40*, 2227–2228.

Van Geet, A. L. (1970) *Anal. Chem. 42*, 679–670.

Warshaw, M. M., & Tinoco, I., Jr. (1966) *J. Mol. Biol. 20*, 29–38.

Nuclear Magnetic Resonance Study on the Exchange Behavior of the NH–N Protons of a Ribonucleic Acid Miniduplex†

Hartmut Fritzsche,‡ Lou-Sing Kan,* and Paul O. P. Ts'o

ABSTRACT: The exchange behavior of the guanine N(1) and uracil N(3) protons in the self-complementary hexanucleotide r(ApApGpCpUpU) has been studied at 5 °C in 80% H_2O/20% D_2O by proton NMR. Under these conditions, the hexanucleotide forms a stable miniduplex. The exchange rate of all Watson–Crick NH protons is unaffected by addition of trifluoroethylamine up to 0.07 M. On the other hand, addition of phosphate buffer, pH 6.9, enhances the exchange rate of the uracil N(3) protons of both terminal and internal A·U base pairs but does not influence the exchange rate of the guanine N(1) protons of the central G·C base pairs. Catalysis by increased phosphate concentrations results in an open-limited rate of the internal A·U base pairs with k_{ex} = 233 s^{-1}, equivalent to a lifetime of 4.3 ms. The proton exchange of the central G·C is regulated by the opening rate of the central core of the miniduplex. On the other hand, the sensitivity of the exchange rate of internal as well as of terminal A·U base pairs can be explained by their reduced lifetime due to end "fraying" and a subsequent catalysis of the exchange process from the opened state. These results suggest that it may be possible to probe labilized parts of RNAs such as tRNA by gradual addition of the exchange catalyst phosphate and to monitor their exchange rates by proton NMR.

The double-stranded, helical structure of DNA is known to exhibit thermally induced local fluctuations, the so-called "breathing" modes (Printz & von Hippel, 1968; McConnell & von Hippel, 1970a,b). Conformational fluctuations may be involved in biologically significant processes, including recognition of DNA base sequences by regulatory proteins in the course of DNA recombination, RNA transcription, and other processes of gene expression, as well as intercalation of drugs, mutagens, and carcinogens.

A number of techniques have been introduced to study the transient opening of double-helical DNA and RNA since the pioneering studies by Printz & von Hippel (1965) and Englander & Englander (1965). Among them, nuclear magnetic resonance (NMR) is a powerful method to obtain information not only on proton exchange rates but also simultaneously on the conformation and environment of the nucleic acids.

In a previous paper we presented a study of the proton exchange of uridine as a function of pH, temperature, and catalyst concentration (Fritzsche et al., 1981) completing the proton-exchange studies on monomeric units of nucleic acids done by McConnell and co-workers as well as other groups (McConnell & Seawell, 1972; McConnell et al., 1972; Cross, 1975; McConnell, 1978; Mandal et al., 1979). In this paper, we present ^1H NMR results on the proton-exchange behavior of a self-complementary hexaribonucleotide helix, r(ApApGpCpUpU)$_2$. The response of the NH–N resonances toward the addition of the potential catalysts phosphate and trifluoroethylamine (TFEA) demonstrates the existence of different mechanisms of the proton exchange for the three different base pairs of this RNA miniduplex. The results are discussed in terms of conformational fluctuations and end "fraying".

Materials and Methods

The hexanucleotide r(ApApGpCpUpU) was synthesized as described previously (Borer et al., 1975). The lyophilized sample was dissolved in phosphate buffer, pH 6.9. The final oligonucleotide concentration in 80% H_2O/20% D_2O was 1 mM (double strand). The starting concentration for the phosphate experiments was 10 mM phosphate. Additional phosphate buffer was added in lyophilized form. 2,2,2-Trifluoroethylamine hydrochloride (TFEA) was obtained from Aldrich Chemical Co. Small aliquots of a 0.16 M TFEA stock solution were added to the hexanucleotide solution containing 0.75 M NaCl, and the pH was adjusted to pH 7.3 after each addition.

The ^1H NMR spectra were recorded on two spectrometers, a Bruker WH-360 spectrometer located at the Mid-Atlantic NMR Facility Center, University of Pennsylvania, Philadelphia, PA, and a Bruker WM-500 spectrometer located at the Southern New England NMR Center, Yale University, New

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Table I: NMR Line Width $\Delta\nu_{1/2}$, Base Pair Lifetime τ, and Base Pair Proton Exchange Rate k_{ex} of the NH-N Protons in the Miniduplex r(ApApGpCpUpU)$_2$ [a]

	$A_1 \cdot U_6$, terminal	$A_2 \cdot U_5$, internal	$G_3 \cdot C_4$, central
$\Delta\nu_{1/2}$ (Hz)	165	48	33
τ (ms)	1.9	6.6	9.6
k_{ex} (s^{-1})	518	151	104

[a] At 5 °C in 80% H_2O/20% D_2O and 0.01 M phosphate at pH 6.9.

Haven, CT. The spectra were recorded at 5 °C (±1 °C) by operating in the continuous wave mode ("correlation spectra") correcting the distortion of the spectra as a consequence of fast sweeping through a small frequency range by a special software program (Dadok & Sprecher, 1974).

Results

The assignment and the line width of the NH–N resonances of this self-complementary helix, r($A_1pA_2pG_3pC_4pU_5pU_6$)$_2$, have been studied by Kan et al. (1975) upon thermally induced helix–coil transition. The three ^{1}H NMR lines at 13–14 ppm (referenced to DSS) were assigned to the three different base pairs, in the order of the resonance position from downfield to upfield, $A_2 \cdot U_5$ internal pair, $G_3 \cdot C_4$ center pair, and $A_1 \cdot U_6$ terminal pair. We detected these NH lines at 14.2 ($A_2 \cdot U_5$ internal), 13.5 ($G_3 \cdot C_4$), and 13.2 ppm ($A_1 \cdot U_6$ terminal) in the 360-MHz spectrum of the hexanucleotide at 5 °C (1 mM duplex concentration, pH 6.9). Under these conditions and in the presence of 0.01 M phosphate, the line widths of the three resonances were 48, 33, and 165 Hz, respectively, from downfield to upfield (Table I). We studied the influence of phosphate as well as of TFEA as potential exchange catalysts on the line widths of these miniduplex NH–N resonances at 5 °C and pH 6.9 (phosphate) and pH 7.3 (TFEA), respectively.

Phosphate Addition. Addition of phosphate at pH 6.9 did not influence the line width of the central $G_3 \cdot C_4$ base pairs but broadened the line of the internal $A_2 \cdot U_5$ base pairs (Figure 1). The line corresponding at terminal $A_1 \cdot U_6$ base pairs disappeared by extensive broadening, following the first addition of phosphate. Consequently, phosphate at pH 6.9 is not effective in broadening NH–N lines of the central $G_3 \cdot C_4$ but lines of both the internal and terminal A·U base pairs.

TFEA Addition. Up to 0.066 M TFEA, the line widths of all three NH resonances of the hexamer duplex did not respond to the addition of the catalyst TFEA (Figure 2). Under the condition of these experiments with 0.75 M NaCl at pH 7.3, the linewidth of the internal $A_2 \cdot U_5$ base pairs is similar to that of the central $G_3 \cdot C_4$ base pairs, and the NH–N line of the terminal $A_1 \cdot U_6$ can still be observed throughout the whole experiment up to the highest TFEA concentration.

As found in preliminary experiments, the duplex formation of the hexanucleotide is prevented in the presence of high TFEA concentrations. Therefore, the experiments were limited to TFEA concentrations well below 0.1 M TFEA. The intactness of the duplex in the presence of TFEA up to 0.07 M at 5 °C is indicated by the absence of any changes of intensity, chemical shift, and line width of all three NH resonances.

Discussion

Three-State Model of Hydrogen Exchange, Proton-Transfer Mechanism, and Exchange Catalysis. Before discussing our results, the underlying models and theories should be briefly outlined.

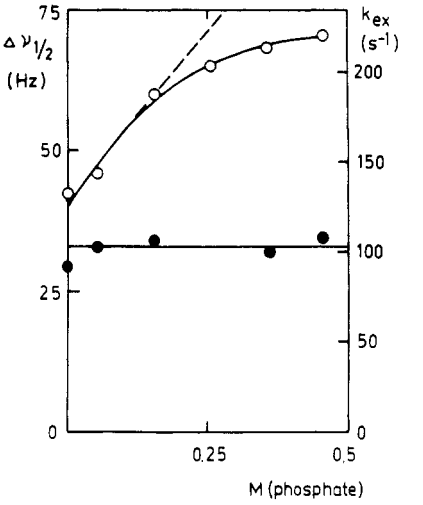

FIGURE 1: Line widths $\Delta\nu_{1/2}$ and overall proton exchange rate k_{ex} of the ring NH–N protons of the RNA miniduplex, r(ApApGpCpUpU)$_2$, at 5 °C and pH 6.9 as a function of added phosphate as an exchange catalyst measured at 360 MHz with correlation spectra. Duplex concentration 1 mM, dissolved in 80% H_2O/20% D_2O containing 10 mM phosphate buffer. (○) U N(3)-H of the internal $A_2 \cdot U_5$ base pairs. Dashed line: Initial slope of the catalytic enhancement of k_{ex} by phosphate. (●) G N(1)-H of the central $G_3 \cdot C_4$ base pairs.

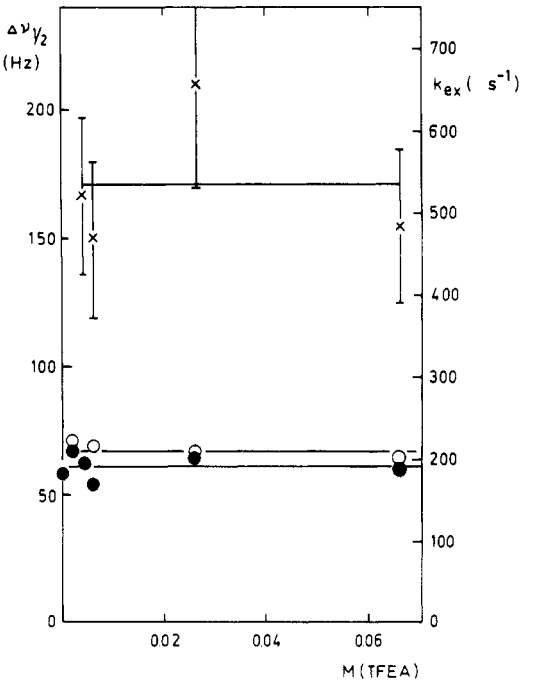

FIGURE 2: Line widths $\Delta\nu_{1/2}$ and overall proton exchange rate k_{ex} of the ring NH–N protons of the miniduplex, r(ApApGpCpUpU)$_2$, at 5 °C and pH 7.3 as a function of added TFEA as a potential exchange catalyst (measured at 500 MHz, correlation spectra). Duplex concentration 1 mM, dissolved in 80% H_2O/20% D_2O containing 0.75 M NaCl. (●) G N(1)-H of the central $G_3 \cdot C_4$ base pairs. (○) U N(3)-H of the internal $A_2 \cdot U_5$ base pairs. (×) U N(3)-H of the terminal $A_1 \cdot U_6$ base pairs.

According to von Hippel & Printz (1965), the NH exchange of ordered nucleic acids can be adequately described by a three-state model based upon the well-known Linderstrom-Lang mechanism (Linderstrom-Lang, 1955). The overall exchange rate k_{ex} of this model is given by

$$k_{ex} = \frac{k_{op}k_{ch}[A]}{k_{cl} + k_{ch}[A]} \quad (1)$$

where k_{op}, k_{cl}, and k_{ch} are the opening, reclosing, and chemical exchange rate constants, respectively, and [A] is the concen-

tration of the exchange catalyst A. The underlying assumptions and limitations of this three-state model were discussed by Hanson (1971).

The line width of the NH NMR resonances reflect the exchange rate k_{ex}. Under the assumption that the excess line broadening is induced merely by proton–proton exchange, the excess line width is related to the exchange rate by

$$\pi\Delta\nu_{1/2} = k_{ex} \quad (2)$$

where $\Delta\nu_{1/2}$ is the additional (excess) line width of the NH resonance line in the ^{1}H NMR spectrum. A more detailed discussion is presented in our previous paper (Fritzsche et al., 1981).

The effect of exchange catalysts can be described in terms of Eigen's theory of proton-transfer mechanism (Eigen, 1964). Again the reader is referred to our previous paper (Fritzsche et al., 1981). By use of pK_As of ~9.5 for the protonation of both G N(1) and U N(3) at 5 °C (Ts'o, 1974) as well as 6.8 and 5.6 for phosphate and TFEA, respectively, the corresponding calculated k_{ex}s (eq 6 of our previous paper) are

$$k_{ex} = (10^{10})(10^{6.8-9.5})[A] = 10^{7.3}[A]$$

for phosphate and

$$k_{ex} = (10^{10})(10^{5.6-9.5})[A] = 10^{6.1}[A]$$

for TFEA where [A] is the concentration of the catalyzing species.

The Exchange Rate of the Central G_3·C_4 Base Pairs Is Limited by the Helix–Coil Interconversion Rate. A lack of response of the observed exchange rate to the addition of catalyst would favor mechanisms with $k_{cl} \ll k_{ch}$, i.e., eq 1 results in

$$k_{ex} = k_{op} \quad (3)$$

Thus, for G_3 N(1)-H, the limiting rate is the helix–coil interconversion rate k_{op}, according to eq 3. We can conclude that the line width of the G N(1)-H resonance of the miniduplex will be determined predominantly by the helix lifetime $\tau = 1/k_{op}$ which we find in the range of 10 ms (Table I). According to the three-state model, there is no reason to expect the G_3 N(1)-H line width to respond to exchange catalyst, if the k_{op} is unaffected by the catalyst. This is supported by our findings that both phosphate and TFEA (<0.07 M) do not affect the G N(1)-H line width (Figures 1 and 2). Mandal et al. (1979) found neither a denaturing effect nor an increase of the U N(3)-H exchange rate of poly(rA)·poly(rU) even at 2.5 M TFEA.

Extensive Catalysis of the A·U Base Pair Exchange Rate Is Limited by Their Opening Rate. On the other hand, both terminal and internal A·U base pairs respond to phosphate as exchange catalyst but not to low concentrations of TFEA which is a weaker catalyst due to its lower pK_A' value. In this case, the results favor mechanisms with $k_{cl} \gg k_{ch}$ of the simple three-state model, resulting in

$$k_{ex} = \frac{k_{op}}{k_{cl}} k_{ch}[A] = Kk_{ch}[A] \quad (4)$$

Further enhancement in exchange by addition of catalyst increases k_{ex} to a value limited by the helix opening rate k_{op} at infinite catalyst concentration. In support of this notion, the observed exchange rate k_{ex} of the internal A_2·U_5 base pairs increases hyperbolically with increasing phosphate concentration; in a $1/k_{ex}$ vs. $1/[A]$ plot, the data result in the predicted straight line (Mandal et al., 1979) with a limiting pseudo-monomolecular opening rate of 232 s^{-1} (Figure 3). This value corresponds to a lifetime τ of 4.3 ms. The catalytic

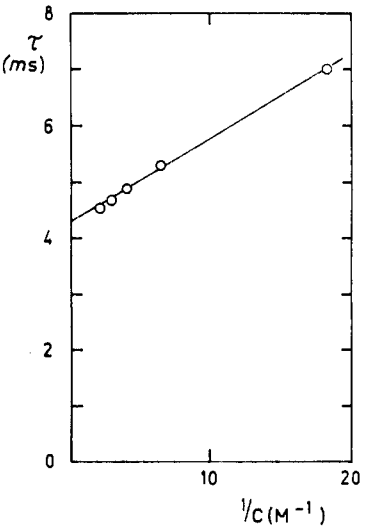

FIGURE 3: Plot of the lifetime τ of the internal A·U base pairs in the duplex of r(ApApGpCpUpU)$_2$ vs. $1/C$ where C is the concentration of the phosphate buffer, pH 6.9, at 5 °C under the assumption $\tau \simeq (\pi\Delta\nu_{1/2})^{-1}$.

effect of phosphate on the A_2·U_5 exchange rate is given by the quantity of 386[A] s^{-1} as obtained from the initial slope of the hyperbolic curve in Figure 1. [A] is the concentration of the active species HPO_4^{2-} which amounts to 50% of the total phosphate concentration at pH ≃ pk_A' (6.8). This enhancement of the proton exchange rate is 4–5 orders of magnitude less than expected by Eigen's theory which would result in $10^{7.3}$[A] s^{-1} as shown above. Similarly, Hilbers & Patel (1975) found the effect of phosphate on the exchange rate of A·T base pairs of the miniduplex of d(ApTpGpCpApT) much lower than expected. They discuss the possibility of repulsion between the negatively charged nucleic acid backbone and the double negative charge of the phosphate ion. Their argument is supported by earlier findings of McConnell & von Hippel (1970a) that negatively charged catalysts are less effective than neutral ones.

The expected response of k_{ex} of the internal as well as the terminal A·U base ring NH protons to the catalyst TFEA may be unobservable under our conditions. By Eigen's theory, one would expect an exchange rate of $10^{6.1}$[TFEA] s^{-1} as shown above. This is more than 1 order of magnitude lower than the phosphate value. Inspection of Figure 1 shows that the enhancement effect of 0.066 M at least on the internal A_2·U_5 base pairs would be small and comparable to the experimental error of the line-width measurement if the catalytic efficiency of the TFEA value is decreased similarly as the phosphate value. Generally, the catalytic effect decreases when the base is incorporated in double-helical oligonucleotides or polynucleotides. Mandal et al. (1979) found the catalytic rate dropped by more than 2 orders of magnitude going from the free base AMP to poly(rA)·poly(rU).

A·U Base Pair Exchange Involves Fraying Intermediates. We can interpret the sensitivity of the A·U exchange rate to catalyst as follows. It is clear from the melting behavior of this and similar miniduplexes (Patel & Hilbers, 1975; Hilbers & Patel, 1975; Kan et al., 1975; Kallenbach et al., 1976) that fraying intermediates are significant in the exchange. The opening rates of the terminal A_1·U_6 as well as the internal A_2·U_5 base pairs are higher than the exchange rate of the opened state to the water as well as the rate of a complete strand dissociation (Patel, 1974; Crothers et al., 1973, 1974; Patel & Hilbers, 1975; Porschke et al., 1973; Ravetch et al., 1974). Thus, a rapid preequilibrium takes place, such that

the overall exchange rate k_{ex} follows the pathway described by eq 4, where K denotes the equilibrium constant for opening and closing of the internal $A_2 \cdot U_5$ base pairs. In this limit, the overall exchange rate should be catalyzed by phosphate buffer which enhances k_{ex} as described by eq 7 in our previous paper (Fritzsche et al., 1981). Thus, in the absence of the destabilization of the duplex, the exchange of the N(3)-H of U at the second position from the end cannot be rate limited by an "all or none" dissociation of the duplex. Intermediate states must intervene.

Lifetime of the Duplex. Mandal et al. (1979) found an opening rate k_{op} of ~ 1.1 s^{-1} for poly(rA)·poly(rU). The overall opening rates of the individual base pairs of our miniduplex are approximately 100–500 s^{-1} (Table I). They differ by 2 orders of magnitude from the corresponding poly(rA)·poly(rU) value. Thus, the difference in the exchange behavior of double-stranded polynucleotides and that of the short miniduplexes reflects the lability of the latter due to fraying of the ends. This holds only for miniduplexes with terminal A·U or A·T base pairs. Fraying is additionally indicated by the lifetime differences of the terminal $A_1 \cdot U_6$ and the internal $A_2 \cdot U_5$ base pairs respectively (Table I). The lifetime of the central $G_3 \cdot C_4$ base pairs (9.6 ms at 5 °C, Table I) agrees well with the lifetime of 17 ms (at 20 °C) of the miniduplex d(ApTpGpCpApT) studied by Hilbers & Patel (1975).

Strikingly, the lifetime of $G_3 \cdot C_4$ base pairs (9.6 ms) is not significantly lowered even at 0.5 M phosphate (Figure 1). The question arises whether this result reflects merely the well-known higher stability of G·C compared with A·U base pairs or that $G_3 \cdot C_4$ base pairs are protected against catalyzing agents simply by their central position which suppresses the influence of end fraying. This observation raises the possibility that catalysts such as phosphate can be employed as a probe of the terminal base pairs in molecules such as tRNA which has many short helical segments.

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Registry No. r(ApApGpCpUpU), 42757-05-1; 2,2,2-trifluoroethanamine, 753-90-2; phosphate, 14265-44-2; guanine, 73-40-5; uracil, 66-22-8.

References

Borer, P. N., Kan, L.-S., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4847–4863.

Cross, D. G. (1975) *Biochemistry 14*, 357–362.

Crothers, D. M., Hilbers, C. W., & Shulman, R. G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2899–2907.

Crothers, D. M., Cole, P. E., Hilbers, C. W., & Shulman, R. G. (1974) *J. Mol. Biol. 87*, 63–88.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243–248.

Eigen, M. (1964) *Angew. Chem., Int. Ed. Engl. 1*, 1–19.

Englander, S. W., & Englander, J. J. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 370–378.

Fritzsche, H., Kan, L.-S., & Ts'o, P. O. P. (1981) *Biochemistry 20*, 6118–6122.

Hanson, C. V. (1971) *J. Mol. Biol. 58*, 847–863.

Hilbers, C. W., & Patel, D. J. (1975) *Biochemistry 14*, 2656–2660.

Kallenbach, N. R., Daniel, W. E., & Kaminker, M. A. (1976) *Biochemistry 15*, 1218–1224.

Kan, L.-S., Borer, P. N., & Ts'o, P. O. P. (1975) *Biochemistry 14*, 4864–4869.

Linderstrom-Lang, K. U. (1955) *Spec. Publ.–Chem. Soc. 2*, 1–2.

Mandal, C., Kallenbach, N. R., & Englander, S. W. (1979) *J. Mol. Biol. 135*, 391–412.

McConnell, B. (1978) *Biochemistry 17*, 3168–3176.

McConnell, B., & von Hippel, P. H. (1970a) *J. Mol. Biol. 50*, 297–316.

McConnell, B., & von Hippel, P. H. (1970b) *J. Mol. Biol. 50*, 317–332.

McConnell, B., & Seawell, P. C. (1972) *Biochemistry 11*, 4382–4392.

McConnell, B., Raszka, M., & Mandel, M. (1972) *Biochem. Biophys. Res. Commun. 47*, 692–698.

Patel, D. J. (1974) *Biochemistry 13*, 2396–2402.

Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2651–2656.

Pörschke, D., Uhlenbeck, O. C., & Martin, F. H. (1973) *Biopolymers 12*, 1313–1335.

Printz, M. P., & von Hippel, P. H. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 363–369.

Printz, M. P., & von Hippel, P. H. (1968) *Biochemistry 7*, 3194–3206.

Ravetch, J., Gralla, J., & Crothers, D. M. (1974) *Nucleic Acids Res. 1*, 109–127.

Ts'o, P. O. P., Ed. (1974) in *Basic Principles* in *Nucleic Acid Chemistry*, Vol. I, pp 462–466, Academic Press, New York and London.

von Hippel, P. H., & Printz, M. P. (1965) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 24*, 1458.