Cardiac effects of hypoxia in the neotenous tiger salamander *Ambystoma tigrinum*

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Summary

The aquatic form of the tiger salamander *Ambystoma tigrinum* lives in high-altitude ponds and is exposed to a hypoxic environment that may be either chronic or intermittent. In many animal species, exposure to hypoxia stimulates cardiac output and is followed by an increase in cardiac mass. The working hypothesis of the present study was that the hearts of these aquatic salamanders exposed to 10–14 days of 5 % oxygen in a laboratory setting would become larger and would differentially express proteins that would help confer tolerance to hypoxia. During exposure to hypoxia, cardiac output increased, as did hematocrit. Cardiac mass also increased, but mitotic figures were not detected in the cardiac myocytes of colchicine-injected animals. The mass increase was probably due to hypertrophy, although a very slow rate of hyperplasia cannot be ruled out. Representational difference analysis indicated that at least 14 mRNAs were expressed in hearts from the hypoxic animals that were not expressed in hearts from normoxic animals. The differentially expressed genes were cloned and sequenced and confirmed as coming from the ventricles of the hypoxic salamanders. Genes differentially expressed include mitochondrial genes and genes for elongation factor 2, a protein synthesis gene. The mechanical performance of buffer-perfused hearts isolated from normoxic and hypoxic animals did not differ. Acute responses to hypoxia were also measured. The rate of oxygen consumption of unanesthetized salamanders in metabolism chambers decreased when chamber oxygen concentration was reduced below 12 % oxygen. At a chamber oxygen concentration of 4–6 %, the rate of oxygen consumption of the salamanders was reduced to approximately one-third of the normoxic rate.

Introduction

Tiger salamanders (*Ambystoma tigrinum*) inhabit ponds in western USA at altitudes up to 3600 m (Sexton and Bizer, 1978). Some of these ponds are permanent and hold salamanders that retain their gills as breeding adults (neoteny). Associated with these high altitudes is a low ambient P_{O_2} in both air and water compared with sea level. Some of these ponds are eutrophic and support photosynthetic organisms that produce oxygen during the day to increase the water P_{O_2}, but their nocturnal respiration decreases the pond P_{O_2} to near zero just prior to sunrise. These high-altitude ponds present the additional challenge of being ice-covered for much of the year, thus limiting the salamanders access to aerial respiration.

It is thought that neotenous salamanders use cutaneous and gill gas exchange at relatively high oxygen tensions and progress to air-gulping and lung ventilation when oxygen tensions become low (Guimond and Hutchison, 1972). Zwemer and Prange (1990) studied adaptations to chronic hypoxia in neotenous *Ambystoma mexicanium* in a laboratory setting. Animals that had been raised since birth at an equivalent altitude of 4000 m (hypoxia-adapted) had a greater underwater rate of oxygen consumption at P_{O_2} values above 135 mmHg (18.0 kPa) than their normoxic counterparts. However, the hypoxia-adapted animals had a reduced rate of oxygen consumption at P_{O_2} values below 40 mmHg (5.32 kPa) compared with the normoxic animals, demonstrating that exposure to chronic hypoxia results in adaptations that are beneficial to living in a low-oxygen environment. Zwemer et al. (1993) exposed *Ambystoma mexicanum* to 14 days of reduced oxygen level and found no differences between the exposed and normoxic animals in a subsequent challenge to progressive hypoxia. During the progressive hypoxia, animals from both groups relied more on air-breathing than on gill ventilation at the reduced oxygen tension and both had increased hematocrits following exposure to a P_{O_2} of 5 mmHg (0.7 kPa).

Many species of vertebrates have cardiac myocytes that are terminally differentiated and do not re-enter cell division, although recent evidence casts doubt on this long-held

assumption (Beltrami et al., 2001). Some species of adult salamander do have cardiac myocytes that undergo cell division in cell culture, and the newt *Notophthalmus viridescens* has been studied extensively (Mantz et al., 1998). Preliminary studies from this laboratory indicated that adult tiger salamanders exposed to 5 % oxygen for a period of 11 days increased their cardiac mass compared with matched normoxic salamanders. Two-dimensional protein gels derived from the hearts of hypoxic and normoxic salamanders showed that hypoxic hearts expressed at least eight proteins that were not expressed in the hearts of normoxic animals (McKean et al., 2001). Since cardiac mass increased during hypoxic exposure, we were interested in learning whether the increase in mass was accomplished through hyperplasia or hypertrophy or combination of the two mechanisms. Further, we were interested in any physiological changes that might be occurring in the heart as a result of differential gene expression or post-translational protein modification. The working hypothesis of this study was that there is differential cardiac gene expression in adult salamanders exposed to hypoxia that results in changes in physiological function and cellular hyperplasia.

Materials and methods

Adult salamanders (*Ambystoma tigrinum* Green, 1825; body mass 70–225 g) were purchased from Charles D. Sullivan Co., Inc. (Nashville, TN, USA) and housed in 60 l aquaria in a laboratory at a temperature of 19–21 °C and with a natural photoperiod. They were fed live goldfish or chopped beef. Experiments were conducted with the approval of the University Animal Care and Use Committee. Salamanders were exposed to chronic hypoxia by covering the aquaria with Plexiglas and bubbling the water with a mixture of compressed air and N_2. The 3 cm layer of gas overlying the aquarium water was sampled throughout the day, and the gas mixture was adjusted so the oxygen content was approximately 6 % (range 4.5–9.0 %). Hypoxic salamanders were kept in the aquarium for up to 14 days, and normoxic salamanders were housed in an identical aquarium exposed to room air. During hypoxia, fresh water was pumped into the hypoxic and normoxic aquaria and stale water was removed at the same rate to reduce the effect of waste build-up. Aquarium filters were also utilized. Animals were fasted for several days before beginning the hypoxia to minimize contamination of the aquarium water. At the end of the hypoxic period, the animals were removed from the aquaria, injected with 0.2 mg of heparin, weighed and anesthetized by immersion in 25 mg ml^{-1} 3-aminobenzoic acid ethyl ester. Anesthesia was established by confirmation of the loss of a righting reflex and failure to withdraw a limb in response to a pinching stimulus.

Buffer-perfused hearts

In situ buffer perfusion of the heart was performed by inserting an input cannula into the sinus venosus and an output cannula into the conus arteriosus (McKean et al., 1997). Other vessels entering and leaving the heart were tied off using surgical silk. Pressure was measured using a World Precision Instruments, Inc. (Sarasota, FL, USA) blood pressure transducer and transbridge amplifier. Flow in the output catheter was measured using a Transonic Systems (Ithaca, NY, USA) flow probe (type 2N). Pressure and flow signals were acquired and displayed on a microcomputer using a DASH-8 A/D board (MetraByte Corp, Stoughton, MA, USA) and Labtech Notebook software (Laboratory Technologies Corp, Wilmington, MA, USA). Flow and pressure calibrations were performed at 20 °C. Buffer used to perfuse the heart had the following composition (in mmol l^{-1}): NaCl, 110; KCl, 1.88; CaCl$_2$, 1.8; NaH$_2$PO$_4$, 0.07; glucose, 5.6; NaH$_2$PO$_4$ was added to adjust the pH to 7.8. Flow into the sinus venosus was regulated by adjusting the height of the input reservoir from 1 to 5 cm above the midlevel of the heart. Afterload was adjusted to a value of 20 cm above the heart for cardiac output determinations and up to the maximum height for which a cardiac output could be generated to determine 'fail height'.

Whole-animal studies

Cardiac output was measured in unanesthetized and minimally restrained salamanders that had a flow probe (type 2S) placed around the conus arteriosus. Animals had been anesthetized during the surgical procedures. Following surgery, the incisions were sutured and the animals were given antibiotics. These animals were placed in a plastic dishpan and heart rate and flow measurements were made before and during hypoxia of up to 7 days duration. It was thought that at least several days of continuous hypoxia would be necessary to initiate the kinds of changes that might be reflective of a chronic response. This would allow sufficient time for physiological adjustments, gene expression and protein synthesis. For studies involving changes in hematocrit and heart mass, exposures of 10–14 days were utilized.

Hematocrit was determined by collecting whole blood in a capillary tube and spinning it for 5 min in a clinical hematocrit centrifuge. Whole-animal oxygen consumption was measured in closed-system respirometers filled with either room air or hypoxic gas mixtures. The cylindrical chambers were two-thirds filled with water so that the salamander could utilize both gill and lung ventilation. A magnetic stir-bar was used to facilitate oxygen equilibration between the gas and liquid phases. When the animal was in place, the chamber was flushed with the experimental gas mixture and the rate of oxygen consumption determined.

Differential gene expression in salamander ventricular tissue was determined using representational difference analysis (Hubank and Schatz, 1999) of ventricular cDNA obtained from hypoxic and normoxic salamanders.

cDNA synthesis

Total RNA was extracted from the ventricles of 15 hypoxic and 15 normoxic salamanders using the Trizol reagent (Life Technologies, Inc., Rockville, MD, USA). The poly(A) mRNA fraction was then isolated from total RNA using oligo poly(T)

paramagnetic beads and a kit purchased from Promega Corp, Madison, WI, USA. Double-stranded cDNA was synthesized from the mRNA using a cDNA synthesis kit purchased from Life Technologies, Rockville, MD, USA.

cDNA representational difference analysis

The driver (normoxia) and tester (hypoxia) cDNA were cleaved with restriction endonuclease *Sau*3A and ligated with *RBam*24/12 dephosphorylated adaptors where the 24-mer was 5′-AGCACTCTCCAGCCTCTCACCGAG-3′ and the 12-mer was 5′-GATCCGTTCATG-3′. The 12-mer adaptor was removed, so the 24-mer served as a primer for the amplification by 25–30 polymerase chain reaction (PCR) cycles to produce the driver and tester amplicons. The adaptors were removed by *Sau*3A digestion, and *Jam*24/12 adaptors were attached to the tester amplicons where the *Jam* 24-mer was 5′-ACCGACGTCGACTATCCATGAACG-3′ and the *Jam* 12-mer was 5′-GATCCGTTCATG-3′. Subtractive hybridization was performed between 4 µg of the non-adaptor driver amplicons and 0.1 µg of the J-adaptor tester amplicons (driver-to-tester ratio of 40:1). The 12-mer was removed by heating, and single-strand products were removed using mung bean nuclease; the first differentially expressed products (DP1) were amplified using PCR and visualized on a 2 % agarose gel. The second differentially expressed products (DP2) were generated by mixing 4 µg of driver and 0.01 µg of tester amplicons (400:1 ratio). DP3 was generated using a driver-to-tester ratio of 4000:1, and the DP4 driver-to-tester ratio was 40 000:1. Difference products were visualized in a 1.8 % agarose gel.

Cloning differentially expressed products

The difference products were removed from gels, and the cDNA was extracted and purified with Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). The difference products were cut with *Sau*3A1 and ligated into the *Bam*HI site of the pBluescript II KS phagemid (Stratagene, La Jolla, CA, USA). Phagemids were introduced into the DH5α strain of *Escherichia coli* and spread on LB-ampicillin/IPTG-X-galactose plates. White colonies were selected, cultured and the phagemids extracted. The presence of inserts was confirmed prior to sequencing by *Bam*HI treatment and gel electrophoresis. Phagemid DNA (400–500 µg) was mixed with 4 µl of dye-labeled nucleotides (Big-Dye), 2 µl of 5×PCR buffer and water to bring the volume to 10 µl and subjected to 25 cycles of PCR. The mixture was dried and cycle-sequenced using an ABI Prism model 377 sequencer (PE Biosystems, Foster City, CA, USA). Sequence similarity searches were performed using the BLAST algorithm (http:www.ncbi.nlm.nih.gov/BLAST/).

Northern hybridization

Total RNA (10 µg of each sample) was electrophoresed on 1 % agarose formaldehyde gels and transferred onto nylon membranes. The blots were hybridized at 68 °C overnight with [α-^{32}P]dCTP-labeled DNA probes generated by representational difference analysis using a PerfectHyp™ Plus hybridization buffer (Sigma). Filters were rinsed as follows: one 5 min wash at room temperature in 2× SSC/0.1 % SDS, two 20 min washes at 68 °C in 0.5× SSC/0.1 % SDS and one 20 min wash at 68 °C in 0.1× SSC/0.1 % SDS. Blots were exposed to Kodak X-ray film using an intensifying screen at –70 °C.

Markers for cell division

Cardiac myocyte mitosis was evaluated by injecting salamanders intraperitoneally with 5 mg of colchicine. The animals had previously been exposed to 9 % oxygen for 3 days. The animals were quickly returned to the hypoxic aquarium; 24 h later, they were anesthetized and the heart was flushed with Telly's fixative, removed from the animal and prepared for light microscopy using haematoxylin and eosin staining.

Cell culture

Tiger salamander ventricular myocyte culture was attempted using techniques described for the newt (*Notophthalmus viridescens*; Mantz et al., 1998). Briefly the technique entailed removing the heart from anesthetized salamander after it had been cleared of blood and subsequently placing it into a modified L-15 medium containing penicillin/streptomycin for 18 h. The ventricle was cut into small pieces, placed into an enzyme solution and agitated for 10 h at 27 °C. The solution was triturated with a 5 ml pipette (diameter 1.5 mm) 3–5 times every 2 h. The suspension was centrifuged at 400 *g* for 5 min and filtered through 125 µm nylon mesh. Cells were examined under a microscope and then preplated in culture dishes for several days. The unattached cells were transferred to new dishes coated with laminin (20 µg ml^{-1}) and were cultured at 25 °C.

Statistical analyses

Data are presented as the mean ± S.D. Statistical evaluation was performed using SigmaStat (SPSS Inc., Chicago, IL, USA), and differences between groups were established using the Student's *t*-test.

Results

Hypometabolism

The rate of oxygen consumption of unanesthetized salamanders placed in metabolism chambers varied inversely with the oxygen concentration of the chamber. Fig. 1 shows that the salamanders (*N*=6) in the low-oxygen group lowered their rate of oxygen consumption significantly in the low-oxygen environment. The same was true for the six salamanders in the medium-oxygen environment, but the three salamanders in the high-oxygen environment did not change their rate of oxygen consumption. The normoxic rate of oxygen consumption among the groups did not differ.

Mechanical performance of the heart

The mechanical performance of the buffer-perfused salamander heart was measured in both normoxic and hypoxic
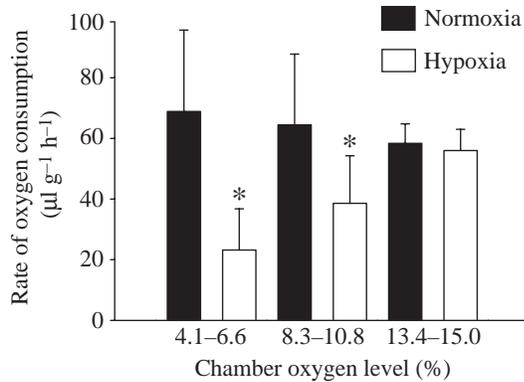
Fig. 1. Rate of oxygen consumption of salamanders in metabolism chambers at three levels of chamber oxygen. An asterisk denotes a significant difference ($P<0.05$) between normoxia and hypoxia. Values are means + S.D. $N=3$ for the high-oxygen group; $N=6$ for the other two groups.

Fig. 3. Frank–Starling relationship for buffer-perfused salamander hearts. Values are means + S.D. $1\,cmH_2O=98.1\,Pa$.

animals at different preloads and afterloads. Fig. 2 shows pressure and flow traces for a buffer-perfused salamander heart. Note that the flow trace lags the pressure trace slightly and that flow reverses and becomes negative during late diastole. The Frank–Starling relationship can be demonstrated in the buffer-perfused salamander hearts. At an afterload of $20\,cmH_2O$ ($2\,kPa$), cardiac output increased as preload was increased from 1 to $5\,cmH_2O$ (0.1–$0.5\,kPa$) (Fig. 3).

The slope of the line relating cardiac output to preload was approximately $20\,ml\,min^{-1}\,g^{-1}\,kPa^{-1}$ increase in filling pressure for both the normoxic ($N=6$) and hypoxic ($N=4$) animals. Several other parameters related to heart performance were recorded from hearts obtained from both normoxic and hypoxic animals. Maximum cardiac output in the hearts from normoxic animals was $30.8\pm9.4\,ml\,min^{-1}\,kg^{-1}$, while the output measured in the hearts from the hypoxic animals was $43.4\pm9.8\,ml\,min^{-1}\,kg^{-1}$ ($N=6$). These differences did not quite achieve statistical significance ($P=0.07$, t-test). The fail height of hearts from the normoxic salamanders was $44\pm12\,cm$ ($N=6$),
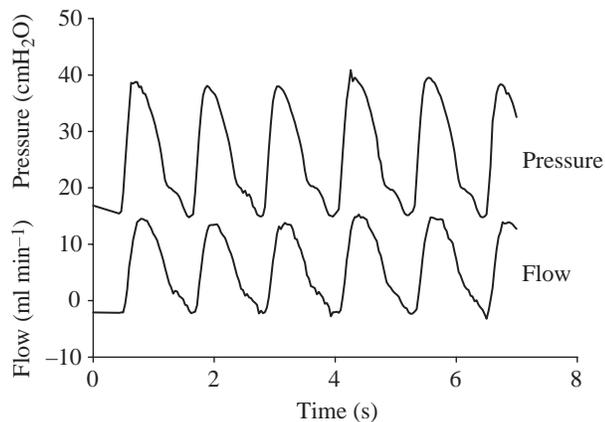
while the fail height of hearts from hypoxic salamanders was $49\pm3\,cm$ ($N=7$). These values also do not differ statistically ($P=0.29$). The maximum cardiac power (P_{max}) in the buffer-perfused hearts of these animals was $1.0\,mW\,g^{-1}$ heart. The equation for cardiac power used is:

$$P_{max}=(Pa-Pv)\times\dot{Q}\times M_v^{-1},$$

where $Pa=2.8\,kPa$, calculated from $[Pd+(Ps-Pd)/3]$, $Pv=0.3\,kPa$, $\dot{Q}=0.083\,ml\,s^{-1}$ and $M_v=0.2\,g$; Pa is arterial pressure, Pv is venous pressure, Ps is systolic pressure, Pd is diastolic pressures, $\dot{Q}$ is cardiac output and M_v is ventricular mass.

Cardiac output and heart rate in unanesthetized and intact animals increased slightly during initiation of hypoxia but then gradually stabilized (Fig. 4). At the termination of hypoxia, there was a brief increase in cardiac output followed by a return to the normoxic value. Cardiac output was elevated or maintained in hypoxic animals unless the oxygen levels were reduced below 1% and held for $10\,min$, which resulted in arrhythmias and cardiac failure.

Hematocrit and cardiac mass

Hematocrit increased in hypoxic animals compared with normoxic animals. Hematocrit during normoxia was 0.414 ± 6.3 ($N=14$) and increased to 0.525 ± 7.5 ($N=8$) during hypoxia ($P=0.002$). Body mass and cardiac output did not differ between the hypoxic and control groups. Body mass was $121\pm29\,g$ in the normoxic animals ($N=15$) and $119\pm34\,g$ in the hypoxic animals ($N=8$). Heart mass to body mass ratio was greater in the hypoxic animals ($P=0.01$): the ratio was $1.8\pm0.5\,mg\,g^{-1}$ in the hypoxic salamanders ($N=8$) and $1.3\pm0.3\,mg\,g^{-1}$ in the normoxic animals ($N=10$).

Differential gene expression

On the basis of two-dimensional gel electrophoresis of proteins from the hearts of hypoxic and normoxic salamanders (data not shown), there was differential gene expression during long-term hypoxia. Agarose gel electrophoresis (Fig. 5) shows the difference products, after 1–4 rounds of subtractive

Fig. 2. Flow and pressure traces for a buffer-perfused heart from a normoxic salamander. Preload was set at $2\,cmH_2O$ ($0.2\,kPa$) and afterload was set at $16\,cmH_2O$ ($1.6\,kPa$). $1\,cmH_2O=98.1\,Pa$.
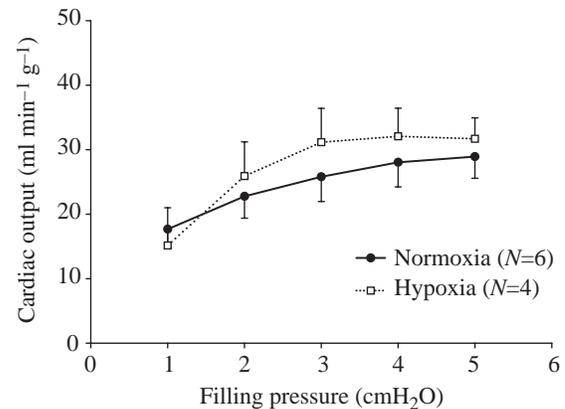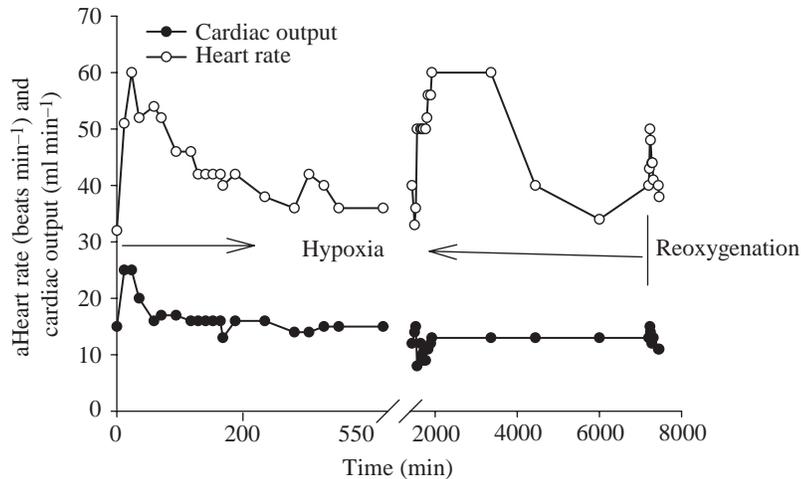
Fig. 4. Heart rate and cardiac output during 5 days of hypoxia in an unanesthetized salamander. The range of oxygen levels during hypoxia was 5–8 %.

hybridization, between hypoxic and normoxic salamander hearts and the amplicons from the hypoxic and normoxic hearts.

The differentially expressed products tend to appear as four bands in the gel, while the DNA in the amplicons has a continuous size distribution. The size of the differentially expressed products ranged from 0.2 to 0.6 kilobase pairs. Several dozen white colonies were picked at random during blue/white screening of the expressed products. cDNA from individual colonies was amplified and sequenced. Fourteen of the gene products from individual colonies were amplified and sequenced, and the gene fragments ranged in size from 394 to 613 bp. Two of the sequenced clones were used to probe hypoxic and normoxic salamander heart cDNA. The probes hybridized only with the cDNA obtained from hypoxic salamander hearts and not the cDNA from normoxic salamander hearts. The sequences of the gene fragments were entered into the http://www.ncbi.nlm.nih database, and a BLAST search was performed. One of the sequences matched that of the cloning vector, pBluescript II KS phagemid. The sequences of the remaining differentially expressed genes
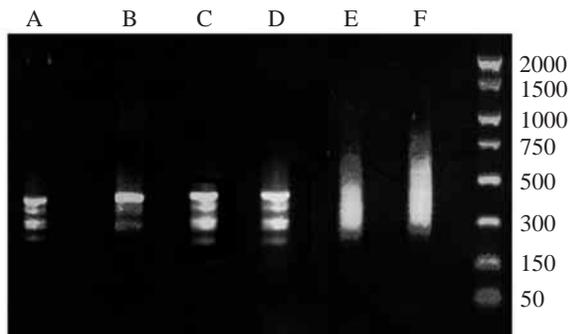

Fig. 5. 1.8 % agarose gel showing amplicons from normoxic (E) and hypoxic (F) salamanders. Lanes A–D show the first, second, third and fourth difference products respectively. The scale shown on the right is in base pairs for fragment size.

were close matches to known genes for either mitochondrial proteins including cytochrome oxidase subunit III or elongation factor 2. The sequence identity ranged from 86 to 98 %. In some cases, there was a comparable degree of match to known genes for both cytochrome oxidase and elongation factor 2. A comparison of the sequences of the cloned genes indicates that here is sequence similarity between three or more of the cloned genes in eight of the genes and little between-gene similarity in six of the cloned genes. The sequences of the gene fragments are presented in the Appendix.

Myocyte isolation and cell culture

Ventricular cardiac myocytes could be readily isolated by enzymatic digestion and separation (Fig. 6). In only one instance were we successful in growing these cells in culture. After plating, the cells began to dedifferentiate, lose their striations and take on a rounded appearance.

Cell division

There was no evidence for cell division in cardiac tissue taken from hypoxic salamanders. Fig. 7 shows that mitotic figures are present in gill epithelia, lung and small intestine in micrographs taken from colchicine-injected hypoxic salamanders. Mitotic figures were not observed in heart tissue from hypoxic animals (N=5).

Discussion

Most of the salamanders used in this study were large adults and in some cases had a body mass approaching 200 g. Large animals were requested from the supplier to facilitate the instrumentation necessary to make measurements from the heart and great vessels. It is likely that they were collected at an elevation ranging from 2000 to 2700 m, but this is not known with certainty. Salamanders were flown to the animal supplier (elevation 120 m) and then to the research laboratory (700 m), where they were held for up to 1 month. Prior exposure to high altitude may influence the experimental results; for example, hematocrit is responsive to changes in altitude. The degree to which prior exposure to altitude influenced the results is not known, but generally the animals had been away from altitude for at least several weeks before experimentation. The level of exposure in the laboratory (approximately 6 % O$_2$) corresponds to an altitude of approximately 8500 m, well above the natural habitat of 2700 m for these animals. Thus, the hypoxic stress placed on the animal in the laboratory would be substantial even if the animals had been residing at their natural altitude habitat.

The neotenous form of these salamanders has been shown to be more hypoxia-tolerant than the metamorphosed form (Branch and Altig, 1983). By living in permanent ponds that are free of aquatic predators such as fish, the neotenous form
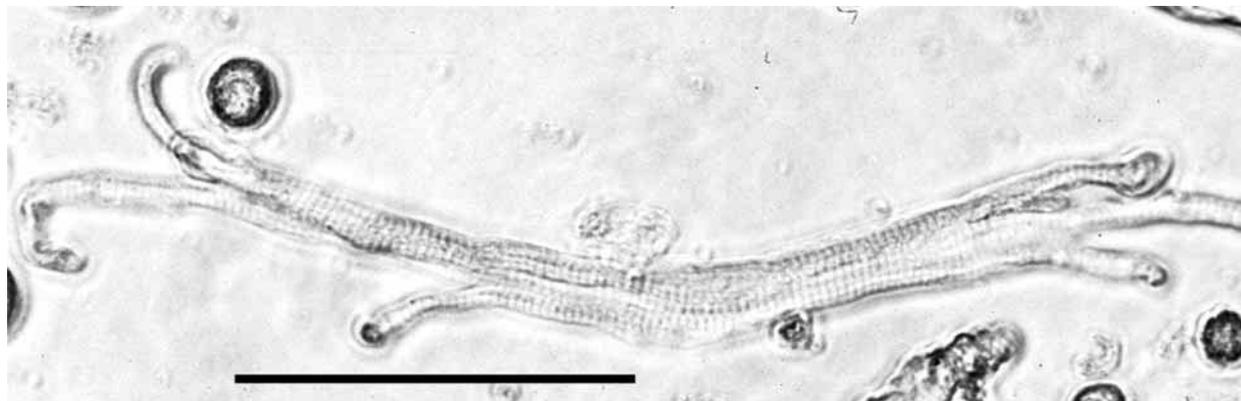
Fig. 6. Ventricular cardiomyocytes from salamander. Two or three myocyes are visible and are incompletely separated. Scale bar, 100 μm.

of the salamander has a distinct advantage over the metamorphosed form (Sexton and Bizer, 1978). The latter is subjected to greater temperature extremes and pressure from aerial and terrestrial predation but has the advantage that it is able to colonize new ponds.

The tiger salamanders used in this study demonstrated decreased rates of oxygen consumption at oxygen concentrations that were less than approximately 13 %, which corresponds to an approximate altitude of 3500 m and would be at the upper limit for these animals. Sheafor et al. (2000) and Gatz et al. (1974) reported that dusky salamanders have a lowered rate of oxygen consumption during hypoxia and the rates of oxygen uptake compared favorably with those recorded in the present study.

Cardiac output measurements in unanesthetized tiger salamanders indicated that a small initial increase in cardiac output was associated with hypoxia. Sheafor et al. (2000) recorded an increase in heart rate during hypoxia at all but the most severe hypoxic challenges in dusky salmanders. Gamperl et al. (1999) also recorded a small increase in cardiac output in unanesthetized toads (*Bufo marinus*) subjected to hypoxia. It thus appears that there is an increase in the activity of the heart under moderate hypoxic condition at a time during which oxygen consumption is reduced compared with normoxia.

The cardiac power development of the buffer-perfused heart of the salamander was $1.04\,\text{mW}\,\text{g}^{-1}$ heart. This compares favorably with the value of $0.8\,\text{mW}\,\text{g}^{-1}$ measured in the heart of *Bufo marinus* (McKean et al., 1997) and $1.5\,\text{mW}\,\text{g}^{-1}$ in the adrenaline-stimulated turtle heart (Farrell et al., 1995). The slope of the Frank–Starling curve in the salamander was $20\,\text{ml}\,\text{min}^{-1}\,\text{g}^{-1}\,\text{kPa}^{-1}$ compared with $50\,\text{ml}\,\text{min}^{-1}\,\text{g}^{-1}\,\text{kPa}^{-1}$ in the toad heart. The reason for the difference in slopes is not known, but it indicates that the salamander heart is less sensitive to stretch-induced increases in cardiac output than the toad heart.

Exposure to hypoxia of more than several days duration resulted in an increase in cardiac mass. The signal for an increase in heart size was probably an increase in cardiac output or cardiac work associated with pumping blood with a higher hematocrit. Increases in work are known to induce increases in cardiac mass and protein expression (Katz, 1992).

The larger hearts did not, however, develop a statistically larger maximum cardiac output during buffer perfusion, nor were they able to achieve a statistically greater fail height than the smaller hearts from the normoxic animals.

Failure to detect mitotic figures in the hearts of colchicine-injected and hypoxia-exposed animals supports hypertropy rather than hyperplasia as the cause of the increase in cardiac mass. Very low levels of cell division might not have been detected using the colchicine technique. If very low levels did occur, the number of dividing cells must be very small since no mitotic figures were detected among the several thousand heart cells examined. However, mitotic figures were readily found in the gill, intestine and lung tissues taken from colchicine-injected salamanders. Direct measures of hypertropy such as increased expression of specific hypertrophy markers were not attempted, so the suggestion that hypertrophy is the primary cause of cardiac mass is based on the process of elimination of hyperplasia as a major cause. Cardiac cell division under cell culture conditions in adults has been described in other salamander species (Mantz et al., 1998) and we observed it in one instance, but were not able to document it photographically.

Differential cardiac gene expression during hypoxia was evident by virtue of the 14 different mRNAs expressed in hearts from hypoxic animals compared with control animals. On the basis of extensive reports in the literature (e.g. Gracey et al., 2001), we expected to see increases in mRNA for proteins such as erythropoietin, glucose transporters and glycolytic enzymes. It is likely that these mRNAs were differentially expressed in the hearts from the hypoxic animals but were not selected during the screening process. Instead, differentially expressed genes fell into two groups: (i) those involved in mitochondrial gene expression and (ii) those involved in protein synthesis (elongation factor 2). These messages were probably selected because they were more abundant than other messages. Since there was a considerable increase in cardiac mass associated with hypoxia, it is not surprising that additional protein synthesis was necessary. Increases in mitochondrial gene expression in hypoxic cardiac tissue were initially unexpected since the mitochondrion is an oxygen consumer and, during hypoxic exposure, oxygen
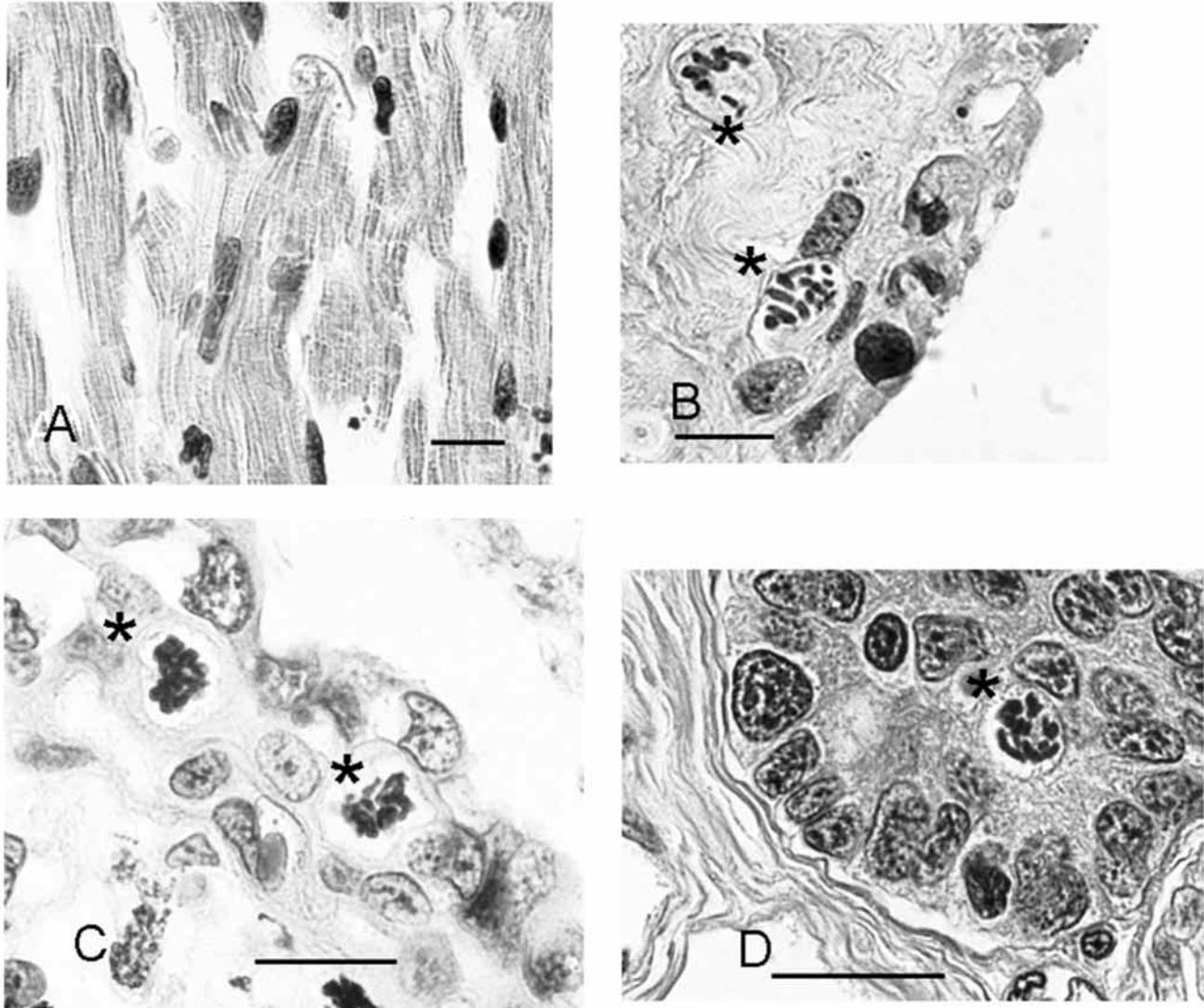
Fig. 7. Micrographs from (A) heart, (B) gill, (C) lung and (D) ileum of hypoxia-exposed salamanders that had been injected with colchicine. The asterisks indicate nuclei in which chromosomal separation has been arrested by the colchicine administration. Scale bars, 20 μm.

conservation was expected. As cardiac mass increased during the hypoxic exposure, it is likely that new mitochondria were also synthesized. Storey (1999) has reported that exposure of adult freshwater turtles to acute hypoxia results in increases in the expression of several mitochondrial genes including those for cytochrome *c* oxidase subunits. The significance of the increases in expression of these mitochondrial genes in the turtle is unclear; however, Storey (1999) suggests that they may somehow participate in a more efficient utilization of the limited oxygen associated with hypoxic exposure. It was not reported, nor was there sufficient exposure time, to determine whether cardiac mass increased in these animals. Bailey and Driedzic (1996) reported decreases rather than increases in protein synthesis in isolated turtle hearts that had been exposed to several hours of hypoxia compared with normoxic turtle hearts. During the hypoxic exposure, mitochondrial protein synthesis was reduced even more than total ventricular protein

synthesis. The hearts in that study were hypodynamic because cardiac power development was considerably less than the power development of the salamander hearts in the current study and in other studies using turtle hearts (Farrell et al., 1994). Because the hearts were under no stimulus to beat more forcefully, there was probably no signal for increasing cardiac mass. However, the oxygen levels achieved in the buffer-perfused turtle hearts were probably much lower than those encountered by the salamander hearts during the extended hypoxic exposure, and severe hypoxic *per se* may initiate a signal to curtail protein synthesis (Land and Hochachka, 1995).

Common mechanisms shared among vertebrates to combat the deleterious effects of hypoxia are cardiovascular and respiratory adjustments that attempt to maintain an adequate flow of oxygen to the respiring tissues. Some vertebrates, such as turtles (Hicks and Wang, 1999), neonatal dogs (Rohlicek et al., 1998) and frogs (West and Boutilier, 1998), enter a state of

hypometabolism in response to environmental hypoxia. In the present study, tiger salamanders exhibited hypometabolism in response to acute hypoxia of less than 13 % inspired oxygen in the laboratory setting. The reduction in the rate of oxygen consumption was approximately 60 % in the 4–7 % ambient oxygen environment and 30 % in the 8–11 % oxygen environment. In anesthetized turtles, Hicks and Wang (1999) observed a 30 % reduction in the rate of O_2 consumption during an exposure to 5 % ambient oxygen. The greater reduction in rate of O_2 consumption of the salamander in a 5 % O_2 environment compared with the turtle might reflect a genuine species difference or it could be related to working with an unanesthetized animal. In the unanesthetized salamander, there is sometimes a startle component that lasts for several minutes. This component could be seen in the studies in unanesthetized animals that were fitted with flow probes and used in the cardiac output experiments. When animals that had been undisturbed by humans for several hours were approached by the investigators, there was sometimes a bradycardia that lasted for up to several minutes. Presumably, if this component of the response were present, it would also have been present in the salamanders in the normoxic group shown in Fig. 1, and thus both normoxic and hypoxic groups would have been equally affected. Since the rates of oxygen consumption in our study compared favorably with values obtained in other studies, a startle component either was probably not present or was minor in magnitude. It is likely that the unanesthetized salamanders would retain beneficial cardiopulmonary reflexes that may have been diminished by pentobarbital anesthesia in the turtles. However, movement-associated O_2 consumption would have at an absolute minimum in the anesthetized turtles, while the salamanders made periodic efforts to escape confinement or the hypoxic stimulus.

Exposure to graded hypoxia in a different species of salamander (*Desmognathus fuscus*) resulted in a decline in whole-animal oxygen consumption. Animals exposed to a P_{O_2} of 25 mmHg (3.3 kPa) decreased their rate of O_2 consumption to 30 % of that of controls, and the rate reached a steady state within 5 min of hypoxic exposure. Heart rate in these animals did not change except at the lowest value of 4 mmHg (0.5 kPa) ambient P_{O_2} (Gatz and Piiper, 1979). Sheafor et al. (2000) also studied the dusky salamander and determined that heart rate was stimulated by graded hypoxia and that the threshold value for reducing oxygen consumption was approximately 8–10 % oxygen, a value similar to that found in the current study. The maximum reduction in the rate of oxygen consumption was 66 %, which is also similar to the value determined in the present study.

A reduction in the O_2 demand of the organism has an obvious benefit during hypoxic exposure. Hicks and Wang (1999) suggest that hypoxic hypometabolism represents a regulated response that might, for example, involve a change in status of ATPases or ion channels rather than a failure to utilize O_2 because of a delivery failure. St-Pierre et al. (2000) suggest that changes in mitochondrial function participate in the regulated response. A regulated response probably occurs in the salamanders because cardiac pumping was maintained at O_2 levels of 5 %. Cardiac failure did not occur until O_2

concentrations had been reduced to under 1 % for almost 10 min, so limitations in O_2 delivery were probably not limiting for the hypometabolism observed in the salamanders. Although flow probes were not utilized during the O_2 consumption studies, blood flow did not decrease during either the acute or the chronic exposure to hypoxia. In the salamander, it appears that there is the dual situation of cardiopulmonary compensation for hypoxia by increased air-breathing with an increase in O_2 delivery in blood fueled by a small increase in cardiac output together with an increase in hematocrit and, presumably, hemoglobin concentration. The increased demand placed on the heart and/or some hypoxia-associated transcription signal results in an increase in cardiac mass and in the expression of specific cardiac gene products that include mitochondrial and protein synthesis genes. These and probably other undescribed mechanisms allow the neotenic tiger salamander to inhabit and flourish in an environment in which oxygen content can present a challenge.

Appendix
Sequences of gene fragments that were differentially expressed in hypoxic salamanders

Sequence 1

```
GGATCCTTCTGGCCGTCCTGCTCAGGTTGTTGCGGAGAC
ACGAAAACGTAAGGGACTTAAAGAAGGCATCCCAGCACTTG
ACAACTTCCTGGACAAATTGTAGAACCAACGCAGTCCTCAG
TACCAACTGAACCAAACAAACGGTCATTGGACTCTTGAAAA
TGTAGCCATCGTAACGTGGAGCTGCCGGCACGATTGTTAGA
CTGCAACAACCATTTGCCTTGCATTTGAAGCGGAGCAACTT
GATTTCCCGAGTACATTGGTATTCAGCCTTATAGGTTTTTC
TGTGTTGCACATTATTTTTGGGGGGCACGCAAGGTGCCGTA
AAGCAAAACATTTCCACTTGATCAACATTCTTTGTAGCAAC
TGGCTTCCACGGCCTTCACGTTATTATT
```

Sequence 2

```
GGATCCCGAAGACTCCATCGTCGCTGCCTTCAAGTTGTT
AGACCCCAATGGCACTGGAAATGTTAAAAAAGATGAGTTGA
AACTACTCCTGATACTCGGTGAGAGGCTGGAGAGTGCTCGT
TCATGGACGTTGACTTTTCCTCTGAAGTAACGGCTGCCCTT
CGTGTCACAGATGGTGCTCTGGTGGTTGTAGACTGTGTATC
TGGCGTCTGTGTACAGACTGAAACGGTGCTACGTCAGGCCA
TTGCAGAGAGAATTCGTCCAGTATTGATGATGCCCCCTCGG
ATAGCACAGTTGCCTAGGCAACTGTGCTATCCGAGGGA
```

Sequence 3

```
GGATCCGAACCGTTAACGTTCTCGCCGAATAGCGACAGG
AAGATCACCGCCCTGGCCCCCAACACCATGAAGATCCCGAA
GACTCCATCGTCGCTGCCTTCAAGTTGTTAGACCCCAATGG
CACTGGAAATGTTAAAAAAGATGAGTTGAAACTACTCCTGA
TGACCCAAGCTGACAAGTTCTCAGACGAAGAGGTGGAGCAG
ATGTTTGCTGTAACGCCAATCGATGTGGCGGGAAACATTGA
CTACAAGTCACTGTGTTACATTATCACCCACGGAGATCATT
ATTCCTATCTGTTTGCCTATTTCGTCAAATTAACTACCACT
TTACATCTAACCACCATTTTAGGTTTGAAGCAGCTGCCTGA
TATTGACATTTCGTTGATGTTGTTTGATTATTCCTTTACGT
CTCCAATCTACTGATGA
```

Sequence 4

CGATCCGTTCATGGATACTGTTCAGGTTTGTTGCGGAGA
CACGAAAACGTAAGGGACTTAAAGAAGGCATCCCAGCACTT
GACAACTTCCTGGACAAATTGTAAAACCAACGCAGTCCTCA
GTACCAACTGAACCAAACAAACGGTCATTGGACTCTTGAAA
ATGTAGCCATCGTAACATGGAGCTGCCGGCAGATTGTTAGG
CTGCAACAACCATTTGCCTTGCATTTGAAGCGGAGCAACTT
GATTTCCCGAGTACATTGGTATTCAGCCTTATAGGTTTTTC
TGTGTTGCACATTATTTTTGGGGGGCACGCAAAGGTGCCGT
AAAGCAAAAACATTTCCACTTGATCCTCCCTCGGATGGCAC
AGTTGCCCGTTCATGGATAGT

Sequence 5

CTCTGGAGCGTGAGGCAGGAGCTGGAACGGGGTGAACTGT
ATATCCCCCACTTGCAGGGAACCTAGCCCATGCCGGGGCCTC
AGTCGATTTAACAATTTTTTCACTTCATTTAGCAGGTGTTTC
ATCTATCCTAGGTGCAATTAATTTTATTACAACCTCAATTAA
TATAAAACCCGCATCAATATCACAATATCAAACCCCTTTATT
TGTTTGATCATTATTCCTATCTGTTTGCCTATTTCGTCAAAT
TAACTACCACTTTACATCTAACCACCATTTTGGGTCTGAAGC
AGCTGCCTGATATTGACATTTCGTTGATGTTGTTTGATTATT
CCTTTACGTCTCAATCTACTGATGAGGGTCAACATTCTTTGT
AGCAACTGGCTTCCACGGCCTTCACGTTATTATTG

Sequence 6

CAATAATAACGTGAAGGGCGTGGAAGCCAGTTGCTACAA
AGAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCT
TGCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTA
TAAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCC
GCTTCAAATGCAAGGCAAATGGTTGTTGCAGTCTAACAATC
TGCCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGT
CCAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGC
GTTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGG
ATGCCTTCTTTAAGTCCCTTACGTTTTCGTGTCTCCGCAAC
AACCTGAGCAGGACGGCCAGAA

Sequence 7

AATAATAACGTGAAGGCCGTGGAAGCCAGTTGCTACAAA
GAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCTT
GCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTAT
AAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCCG
CTTCAAATGCAAGGCAAATGGTTGTTGCAGTCTAACAATCT
GCCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGTC
CAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGCG
TTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGGA
TGCCTTCTTTAAGTCCCTTACGTTTTCGTGTCTCCGCAACA
ACCTGAGCAGGACGGCCAGAA

Sequence 8

AATAATAACGTGAAGGNCGTGGAAGCCAGTTGCTACAAA
GAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCTT
GCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTAT
AAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCCG
CTTCAAATGCAAGGCAAATGGTTGTTGCAGTCTAACAATCT
GCCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGTC

CAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGCG
TTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGGA
TGCCTTCTTTAAGTCCCTTTACGTTTTCGTGTCTCCGCAAC
AACCTGAGCAGGACGGCCAGAA

Sequence 9

GGAAGATATCCGCAGCACCCTGAGGGTCCCCGAAGAACA
ACGCGCGGGGCCCAAAACCCTATTGGGCGCGAACTCACGGA
GGAAGTAATGGCCACAAGGTTGGTACATCAAGGGCATAGCC
GAAGCCTGTGATATACTGACAGCCGGTCCCAAACCGCCCGG
AACTTACGACCCGGGAACCCAAAGTCCAAAGGCCGGGGGGC
CGAATGAGAGAGCCAAGATCGAATGAATAGGGTACACCTGA
CGGGGCCGCACATCGATGCCGGAAAAGCGATTGCACGCCAA
CTAATCCGATCCGGGGGGAAATCCCATATAAGGCCATTGGC
GGCATATGATGCATGAATTGCAATGGCAATCGTACATGACT
GAAAATGGGGGGGGCATTTTACCGATGAACCATGGACCATTC
GGATTGCCATTACCATTTTGGGAAACGATGCATGAACCTGG
AGGAATTCCAGCAGTTAGGCAATTCGTTAGTTACCCAAATG
GAAATTTGCGCGACGACATGATGACG

Sequence 10

CGAGGGGGGGCCCGGTACCCAGCTTTTGTTCCCTTTAGT
GAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTG
TTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAA
CATACGAGCCGGGAGCATAAAGTGTAAAGCCTGGGGTGCCT
AATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG
CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTA
ATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGGGCTCG
GTCGNTCGGCTGCGGGGAGCGGTATCAACTTACTTCAAAGG
CGGGNAATACCGGTATNCACAAGAAACAAGGGGATAACGCA
LJGGAAAGAACATGTGAGCCAAAAAGGCCNGCCAAAANGGC
CANGGAACCCGNAAAAAANGGCCNCGGTTGGCTTGGCGNTT
TTTTCCATTAAGGCTTCCCGGCCCCCCCCTGGGGCGGAGGC
CATTTNNCAAAAAAAAATNNGACCCCCCTTCNAAAAANNTNA
AANAANGGGGGGGGGGGGAAAAACCCCCGNCCNNGGGGACCT
TNTTTAAAAGGAAATNCCCCAAGGGGGGGTTTTCCCCCCCT
GGGGAANACCTTCCCCTTTGGGGGGGGCCTT

Sequence 11

TCCCTCGGATAGCACAGTTGCCTAGGCAACTGTGCTATC
CGAGGGAGCATCATCAATACTGGACGAATTCTCTCTGCAAT
GGCCTGACGTAGCACCGTTTTAGTCTGTACACAGACGCCAG
ATACACAGTCCACAACCACCAGAGCACCATCTGTGACACGA
AGAGCAGCCGTTACTTCAGAGGAAAAGTCAACGTGGCCAGG
GGAGTCGATCCTCCCTCGGATAGCACAGTTGCCTAGGCAAC
TGTGCTATCCGAGGGAGCATCTCCGGGGGTGATAATGTAAC
ACAGGGACTTGTAGCCAATGTTTCCCGCCACATCGATTGGC
GTTACAGCAAACATCTGCTCCACCTCTTCGCTGAGAACTTG
CAGCTTGGGTCATCAGGAGTAGTTTCAACTCATCAGGAGTA
GGTTCAACTCATCCTTTTTAACATTTCCAGGGCCATTGGGG
GGCAAAGTTAATGTGAAGGACGAGGAACCGGAGGGAGATGC
TAAAGGAGGGAAAAGGTCCCATCAACCCCACGGGCCTTCCT
GGGCGCTATCCGCGAAAAGCTTAACGGGTCGGATCAACCTT
CTTTTTGGGCAACCTGGCTTTCAAGGGCTTCACCGGATTATT
G

Sequence 12

```
  AATAATAACGTGAAGGCCGTGGAAGCCAGTTGCTACAAA
GAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCTT
GCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTAT
AAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCCG
CTTCAAATGCAAGGCAAATGGTTGTTGCAGTCTAACAATCT
GCCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGTC
CAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGCG
TTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGGA
TGCCTTCTTTAAGTCCCTTACGTTTTCGTGTCTCCGCAACA
ACCTGAGCAGGACGGCCAGAA
```

Sequence 13

```
  AATAATAACGTGAAGGCCGTGGAAGCCAGTTGCTACAAA
GAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCTT
GCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTAT
AAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCCG
CTTCAAATGCAAGGCAAATGGTTGTTGCAGTCTAACAATCT
GCCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGTC
CAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGCG
TTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGGA
TGCCTTCTTTAAGTCCCTTACGTTTTCGTGTCTCCGCAACA
ACCTGAGCAGGACGGCCAGAA
```

Sequence 14

```
  AATAATAACGTGAAGGCCGTGGAAGCCAGTTGCTACAAA
GAATGTTGATCAAGTGGAAATGTTTTGCTTTACGGCACCTT
GCGTGCCCCCCAAAAATAATGTGCAACACAGAAAAACCTAT
AAGGCTGAATACCAATGTACTCGGGAAATCAAGTTGCTCCG
CTTCAAATGCAAGGCAAATGGNTGTTGCANNCTAACAATCT
GNCGGCAGCTCCACGTTACGATGGCTACATTTTCAAGAGTC
CAATGACCGTTTGTTTGGTTCAGTTGGTACTGAGGACTGCG
TTGGTTCTACAATTTGTCCAGGAAGTTGTCAAGTGCTGGGA
TGCCTTCTTTAAGTCCCTTACGTTTTCNTGTCTCCGCAACA
ACCTGAGCAGGACGGCCAGAAGGATCAACATTCTTTGTAGC
AACGNGGCTTCCACGGCCTTCCGTTATTTT
```

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