



Morphological, clinical and genetic aspects in a family with a novel LAMP-2 gene mutation (Danon disease)

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Received 30 July 2004; received in revised form 13 December 2004; accepted 27 December 2004

Abstract

A family with several cases of severe cardiomyopathy and moderate myopathy is described, affecting two brothers and their cousin as well as their mothers. One boy died of sudden cardiac arrest at 17 years of age. The two brothers were treated with an implantable defibrillator and their mother died suddenly at 40 years of age. Muscle biopsy in males showed vacuolar myopathy in two cases, and no abnormality on standard staining in the third case. Cardiac biopsies showed hypertrophic and vacuolated fibres. Complete absence of LAMP-2 was demonstrated by immunohistochemistry on the vacuolated skeletal and cardiac muscle, but also on the morphologically normal skeletal muscle. Sequencing of LAMP-2 gene showed a novel S157X mutation in exon 4. Danon disease is a rare and potentially lethal cause of hypertrophic cardiomyopathy. Diagnosis can be made by immunohistochemistry performed on cardiac or muscle biopsy, and confirmed by genetic analysis, which also allows for easy family screening and counselling.

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Keywords: Danon disease; LAMP-2; Immunohistochemistry; Cardiomyopathy; Myopathy; Morphology; Genetic

1. Introduction

Hypertrophic cardiomyopathy is a heterogeneous genetic cardiac disease [1], mainly, but not only caused by contractile sarcomeric protein genes mutations [2]. Association with skeletal myopathy may occur, as in Danon disease [3]. This X-linked cardioskeletal myopathy was originally reported as ‘lysosomal glycogen storage disease with normal acid maltase’ [4], and belongs to the autophagic vacuolar myopathies [5]. The disease is caused by primary deficiency of lysosome-associated membrane protein-2 (LAMP-2) [6], one of the proteins thought to protect the lysosomal membrane from proteolytic digestion and to act as a receptor for proteins to be imported into lysosomes [7]. A Swiss

family with six patients (four males and two females) is presented. Histologically, the absence of LAMP-2 was shown for the first time on cardiac biopsies, and also on morphologically normal skeletal muscle and proved to be an important diagnostic tool. A novel mutation in the LAMP-2 gene, located on Xq24 [8], was demonstrated.

2. Case reports

2.1. Case 1

The proband, a boy born in 1976, had normal motor milestones, but some delay in language development. He attended a regular school, but neuropsychological testing showed a total IQ of 76 and his school performance

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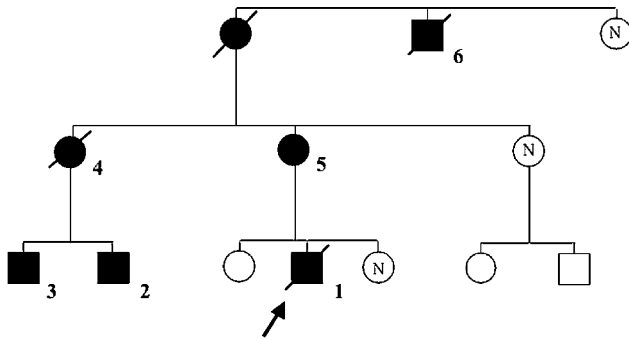


Fig. 1. Family pedigree. The arrow indicates the proband. Males are denoted by squares and females by circles. *N* stands for genetically tested people with normal result.

remained poor. He never did well in sports and fatigued easily. At the age of 13 years, his paediatrician heard a systolic murmur and a chest X-ray showed a cardiomegaly. Further work, revealed a severe left ventricular hypertrophic cardiomyopathy as well as ventricular arrhythmia and medical treatment was started. He was first seen in our center at the age of 6 years. Clinical examination showed diffuse muscular atrophy with mild proximal and axial weakness: the patient could not sit from supine without help of his arms. Deep tendon reflexes (DTR) were present. Serum CK were increased to eight times the norm and EMG was myopathic. Cardiac biopsy and skeletal muscle biopsy from the anterior rectus muscle were performed at 16 years of age. The patient died suddenly 1 year later (Fig. 1).

2.2. Case 2

The patient is the first maternal cousin of patient 1. He walked at the age of 18 months and was later described as clumsy with fatiguability and muscle cramps on exertion with no real progression over time. His school performance and intelligence were both normal. A work up done when he was 8 years of age, just after the death of his cousin, revealed a moderate hypertrophic cardiomyopathy and slightly elevated serum CK to three times normal. At the age of 15 years he experienced episodes of syncope and palpitation. The echocardiogram showed a dramatic progression of the left ventricular hypertrophy. He was admitted to our hospital 1 year later, for cardiac and skeletal muscle biopsies and implantation of a cardiac defibrillator. Clinical examination revealed mild diffuse muscle atrophy without weakness.

DTR's were brisk. Serum CK was eight times the norm. EMG was myopathic, with rare myotonia. Brain MRI was normal.

2.3. Case 3

The patient is the younger brother of case 2. He walked at 18 months, and never complained of fatigue, weakness or

cramps. He initially had a slight language delay, but his school performance and intelligence were both normal. He was 5 years old when his cousin died. His medical work up showed a normal heart, but elevated serum CK (three times normal). While his echocardiogram was still normal at the age of 9 years, a mild cardiac hypertrophy was diagnosed 4 years later. A prophylactic cardiac defibrillator was implanted 1 year later, with a skeletal muscle biopsy performed at the same time.

2.4. Case 4

The patient was the mother of cases 2 and 3. She was diagnosed with a cardiomyopathy and died at the age of 40 years, 1 week before a scheduled surgery for implantation of a cardiac defibrillator.

2.5. Case 5

The patient was the mother of case 1. When last seen at the age of 53 years, she was asymptomatic, but echocardiogram showed slight left ventricular hypertrophy.

2.6. Case 6

The patient is the maternal uncle of cases 4 and 5. He is said to have died at a 'young age' of a cardiac disease.

3. Material and method

3.1. Muscle and cardiac biopsies

Open skeletal muscle biopsies were obtained from the anterior rectus (case 1) and the pectoralis (cases 2 and 3), frozen in 2-methyl-butane, cooled by liquid nitrogen, and stored at -80°C . Ten-micrometer thick sections were stained with haematoxylin–eosin (H&E), periodic-acid-Schiff reagent (PAS), oil-red-O and a modified Gomori stain [9]. Enzyme histochemistry with NADH-tetrazolium-reductase, ATPase at pH 4.2, cytochrome oxidase, succinyl-deshydrogenase, phosphorylase, phosphofructokinase and acidic phosphatase was performed on case 2, according to standard techniques. For immunohistochemistry, the following anti-sera were used on cases 2 and 3: H4B4 (LAMP-2), acetone fixed, dilution 1:80; dystrophin I (rod domain), II (C-terminus) and III (N-terminus) (Novocastra, mouse mAb, clones Dy4/6D3, Dy8/6C5 and Dy10/12B2, 1:20); utrophin I and II (Novocastra, mouse mAb, clones DRP1/12B6 and DRP2/20C5, 1:20); adhalin (Novocastra, mouse mAb, clone Ad1/20A6, 1:200); beta-, gamma- and delta-sarcoglycan (Novocastra, mouse mAb, clones Beta-Sarc/5B1, 35DAG/21B5, Delta-Sarc3/12C1, 1:200); merosin (Chemicon, mouse mAb, catalogue No. MAb1922, 1:2000); laminin 300 kD (Novocastra, mouse mAb, clone Mer3/22B2, 1:80);

spectrin (Novocastra, mouse mAb, clone SPEC1, 1:200); desmin (Dakopatts, code No. M760, mouse mAb, clone D33, 1:50); emerin (Novocastra, mouse mAb, clone 4G5, 1:50); ubiquitin (Zymed, mouse mAb, clone Ubi-1, 1:3); Alzheimer precursor protein A4 (Boehringer, mouse mAb, clone 22C11, 1:5, pre-treatment with formic acid). C5b9 (Dako, mouse mAb, clone aE11.(1), 1:10) was performed on case 2. On case 1, dystrophin I, II, and III, and desmin were used. Standard procedure with the ABCComplex was used.

The cardiac biopsies of cases 1 and 2 were fixed in 4% formaldehyde and embedded in paraffin. Eight-micrometer thick sections were stained with H&E, and immunohistochemistry was performed with LAMP-2 antibodies at a 1:50 dilution.

For conventional transmission electron microscopic study, specimens of skeletal and cardiac muscles of case 2 were fixed in 4% glutaraldehyde buffered with 2.3% cacodylate, and embedded in epoxy resin. Semi-thin sections (0.5–1 μ) were stained with methylene blue and Azur. Ultrathin sections (0.05 μ) were stained with uranyl acetate and lead citrate. Philips electron microscope CM10 was used for examination of the sections, collected on copper grids.

3.2. Genetic analysis

3.2.1. Amplification of LAMP-2

Each exon and the intron–exon junctions were amplified by PCR using the following conditions: 200 ng of DNA, 1.25 μ M of each exon primer, 2% formamide, and 15 μ l of Master Mix (Qiagen, Switzerland) in a final PCR reaction volume of 30 μ l, performed for 35 cycles (1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C) after an initial denaturation step at 95 °C for 5 min and a final 10 min 72 °C extension.

3.2.2. DNA sequencing

PCR products were purified with the High Pure PCR Product Purification Kit (Roche, Switzerland). The sequencing reaction, which included 1 μ l template, 1 pmol sequencing primer and Big Dye Terminators (version 1.0, Applied Biosystems, Switzerland) for a 10 μ l final volume, was performed for 25 cycles (10 s at 96 °C, 5 s at 55 °C, and 4 min at 60 °C) after an initial denaturation step (96 °C for 10 s). The product was purified using a Sephadex G-50 column (Millipore, Switzerland) and sequenced bidirectionally using an ABI Prism 310 genetic analyser and the forward primer.

4. Results

Cardiac biopsies from patients 1 and 2 were performed in the sub-endocardial muscle of the right ventricular septum. No fibrosis, amyloid, inflammation or architectural disarray

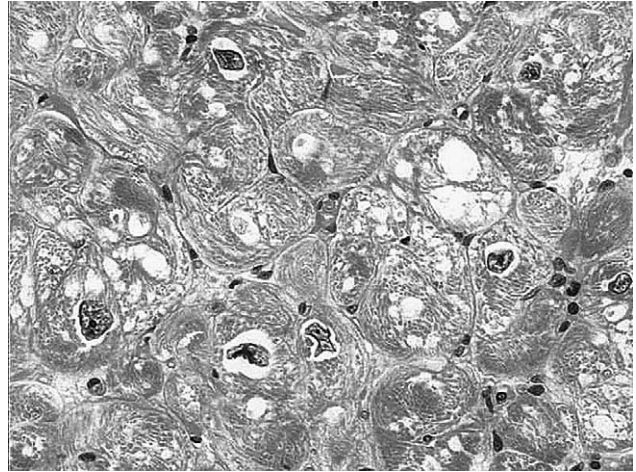


Fig. 2. Heart biopsy of case 1, showing enlarged and vacuolated cardiomyocytes, H&E, original magnification 400 \times .

were observed. The cardiomyocytes were hypertrophic, with enlarged and irregular nuclei, and vacuolated cytoplasm (Fig. 2). Immunohistochemistry showed complete absence of LAMP-2 (Fig. 3). Ultrastructural examination of patient 2 showed numerous vacuoles, containing degenerating mitochondrion, glycogen, small vesicles and granular debris (Fig. 4).

Skeletal muscle biopsies of patient 1 examined with H&E coloration showed no fibrosis or inflammation. Fibre size was normal. Glycogen content was normal on PAS stain. Slightly increased internal nuclei were observed. Few centrally located pale vacuoles were also observed, without granular basophilic material (Fig. 5). Immunohistochemistry against dystrophin showed multiple dystrophin-positive vacuoles in about 10% of the fibres, centrally located (Fig. 6).

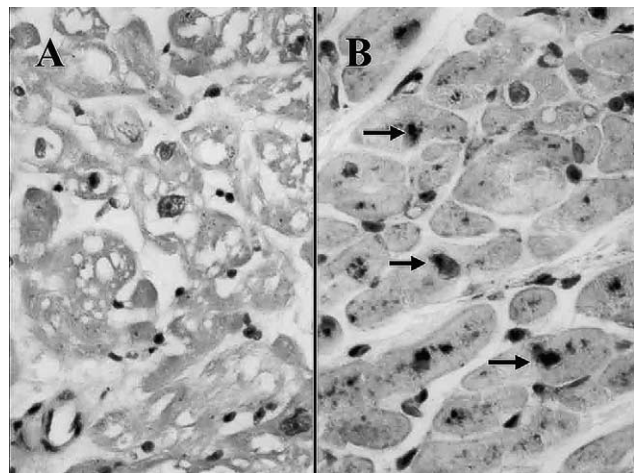


Fig. 3. Heart biopsy. (A) biopsy of case 1, showing complete absence of LAMP-2 expression, immunohistochemistry against LAMP-2, original magnification 400 \times . (B) Control case, showing normal lysosomal LAMP-2 expression (arrows), immunohistochemistry against LAMP-2, original magnification 400 \times .

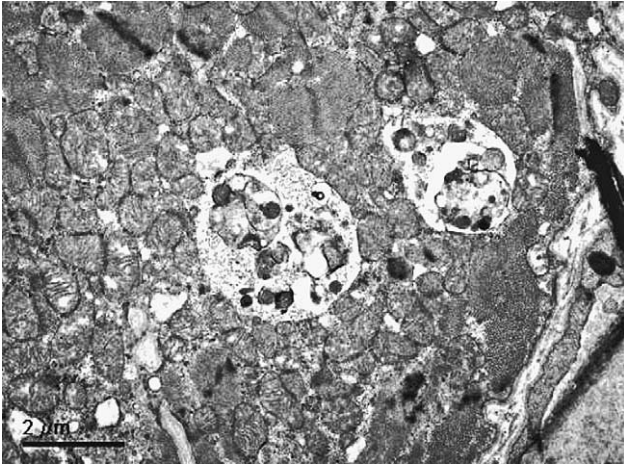


Fig. 4. Ultrastructure of case 2 cardiomyocytes, showing accumulation of autophagic vacuoles, with degenerating mitochondrion, glycogen, small vesicles and granular debris.

In patient 2, H&E examination of skeletal muscle was normal, without vacuoles. Glycogen content was normal on PAS stain. Dystrophin immunohistochemistry revealed small dystrophin-positive vacuoles similar to patient 1 in about 1% of the fibres. These vacuoles were also positive for spectrin, adhalin, beta-, gamma- and delta-sarcoglycan, merosin, ubiquitin. C5b9 was present in some of the vacuoles, but not at the surface of the muscle fibre or in the sarcoplasm. Ultrastructure showed very few small autophagic vacuoles and normal glycogen content.

Complete absence of LAMP-2 was demonstrated in both patients.

In patient 3, H&E examination of skeletal muscle was normal, without vacuoles. Also immunohistochemistry failed to show any vacuole. The only abnormality was complete absence of LAMP-2 expression (Fig. 7).

No morphological examination was performed on patient 4.

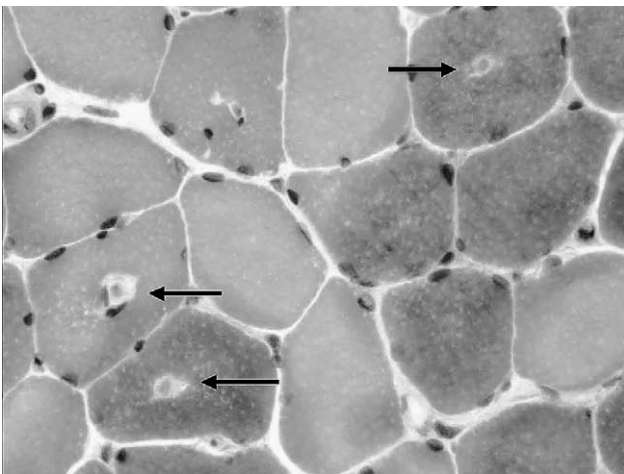


Fig. 5. Skeletal muscle of case 1, showing centrally located pale vacuoles (arrows), H&E, original magnification 400 \times .

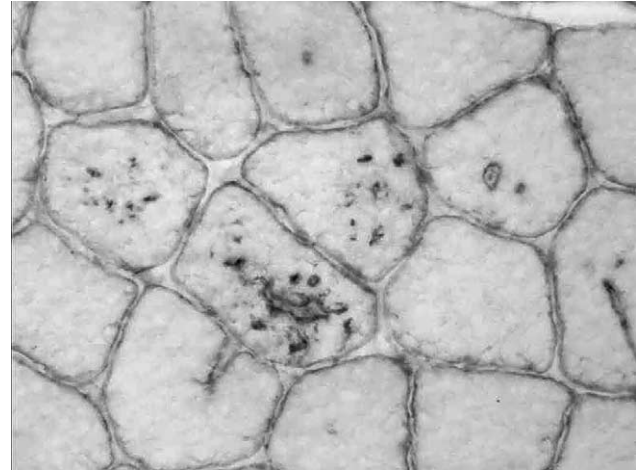


Fig. 6. Skeletal muscle of case 1, showing dystrophin positive centrally located vacuoles, immunohistochemistry against dystrophin I, original magnification 400 \times .

4.1. Genetic analysis

Individual exons of the LAMP-2 gene were amplified from genomic DNA and sequenced directly. NCBI sequence NM_013995 (<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=7669502>; corresponding to Ensembl transcript ID ENST00000007752; www.ensembl.org) was used for comparison. The LAMP-2 cDNA comprises approximately 2600 bases, with the initiation codon at position 136–138. In DNA from case 2, hemizyosity for a 605C>G transversion was found that changed the codon of serine 157 into a stop codon (S157X; Fig. 8). This mutation was also observed in the DNA from his brother (case 3) and at heterozygosity, in their aunt (case 5). It was not found in 100 control chromosomes.

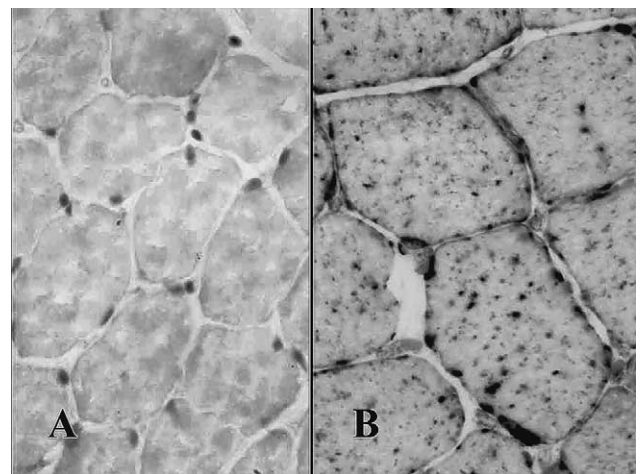


Fig. 7. Skeletal muscle of case 3. (A) Complete absence of LAMP-2 expression, immunohistochemistry against LAMP-2, original magnification 400 \times . (B) Control case, with normal granular lysosomal LAMP-2 expression, immunohistochemistry against LAMP-2, original magnification 400 \times .

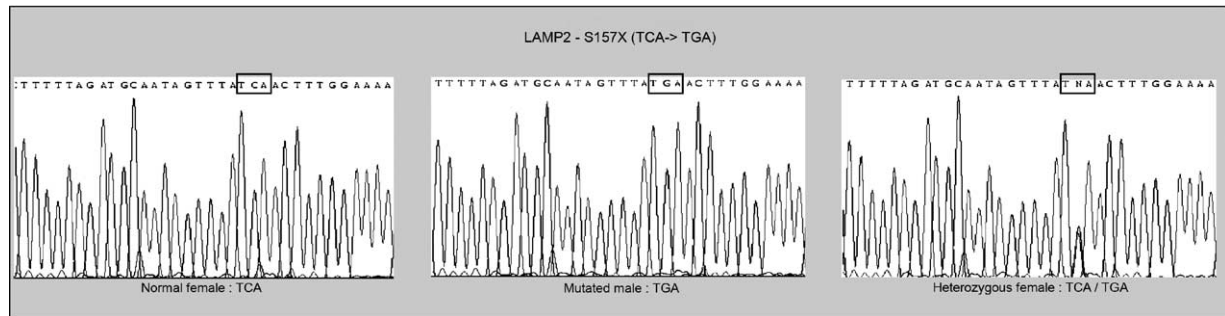


Fig. 8. S157X point mutation is observed in exon 4 of the LAMP-2 gene.

5. Discussion

Since Danon disease was first published, over 20 families have been reported. The family we report is among the largest described with six affected individuals who presented a fairly classical clinical course. Onset was in childhood with fatigability on exertion and elevated CK in the two older boys (Cases 1 and 2) who then had a rapidly progressive hypertrophic cardiomyopathy in early teenage. Cases 2 and 3 sought medical attention before 10 years of age because of the death of their mother and cousin. Thus the progression of disease could be followed prospectively. Case 3 had no complaints and a normal physical examination until 13 years of age when the first echocardiographic signs of hypertrophy were found. However, his serum CK was already elevated when he was first seen at 5 years of age. Mental retardation was only present in patient 1 and was mild. Death at the age of 40 years in case 4 was in accordance with age of death in affected women as seen in previous reports. It is remarkable that her sister, the mother of the proband, was asymptomatic at the age of 53.

Morphologically, the skeletal muscle biopsies showed variable pathological findings among male patients: vacuoles could be seen in about 10% of fibres in patient 1 and only in about 1% in patient 2. No vacuoles were observed in the muscle of patient 3 despite complete absence of LAMP-2 expression. This could be attributed to his young age as the number of vacuoles are known to increase with age (3). In sporadic cases, the absence of vacuoles might contribute to diagnostic difficulty, since they are considered the pathological hallmark of the disease. Therefore, in the appropriate clinical setting, LAMP-2 immunohistochemistry should be performed before ruling out Danon disease. Complete absence of LAMP-2 was also observed in the two performed cardiac biopsies, together with fibres hypertrophy and slight vacuolisation. LAMP-2 immunohistochemistry can thus be performed on skeletal or cardiac muscle, with easy diagnosis.

Danon disease is different from the Xq28 linked vacuolar myopathy [10,11]. This disease is not associated with LAMP-2 deficiency, there is no cardiac involvement,

and complement membrane attack complex is shown on the sarcolemmal surface [11,12]. In our cases, complement membrane attack complex was seen in some of the vacuoles, but not on the fibre surface.

The lysosomal system is the main intracellular mechanism for the turnover of endogenous and exogenous macromolecules. LAMP-2 belongs to the lysosome-associated membrane proteins, which are ubiquitous, highly glycosylated, integral membrane proteins of largely unknown function [13]. How a defect in this protein leads to both severe hypertrophic cardiomyopathy and skeletal myopathy remains to be elucidated. Myocardium hypertrophy may be the result of a compensatory reaction to depressed myocardial contractility. It could also be related to an alteration in cellular metabolism. The inability to maintain ATP (adenosine triphosphate) levels or its utilization has been recently proposed as a possible explanation for the development of hypertrophic cardiomyopathy [14,15]. Such an ‘energy compromise’—term used by Watkins—could be the trigger for hypertrophy in other metabolic disorders such as Fabry disease, Pompe disease and Friedreich ataxia [16].

LAMP-2 locus is located to chromosome Xq24 [8]. Eight different mutations were identified in 10 unrelated patients, in exons 1, 4, 8 and 9, introns 5 and 6, and intron5/exon6 junction [6]. Recently novel mutations in exon 7 [17] and exon 8 [18] were reported. In the family reported here, a novel non-sense mutation in exon 4, S157X, was identified. This mutation predicts the truncation of the protein at amino acid 156. The truncated protein lacks a transmembrane domain. This mutation is analogous to several other mutations reported by Nishino et al. [6] and may be assumed to represent a LAMP-2 null allele. This assumption is reinforced by the absence of LAMP-2 immunoreactivity in the biopsies. It remains to be determined, in premature termination mutations, whether a shortened non-functional protein is synthesized or whether non-sense mediated m-RNA decay prevents protein synthesis. The recent report on the absence of a detectable mutation in blood from the mother of affected sibs points to the possibility of germinal mosaicism: this must be taken into account when investigating on the first generation of affected individuals [19].

Acknowledgements

The H4B4 hybridoma/monoclonal antibody developed by J. Thomas August and James E.K. Hildreth was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

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