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A Population in Limbo: Unisexual Salamanders (Genus *Ambystoma*) Decline Without Sperm-donating Species

James P. Bogart[1,4], Jessica E. Linton[2], and Al Sandilands[3]

[1]Department of Integrative Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
[2]Natural Resource Solutions Inc., 225 Labrador Drive, Unit 1, Waterloo, Ontario N2K 4M8, Canada
[3]Gray Owl Environmental Inc., 1356 Lockie Road, Branchton, Ontario N0B 1L0, Canada
[4]Corresponding author, email: jbogart@uoguelph.ca

***Abstract.*—In all Canadian populations, Jefferson Salamanders (*Ambystoma jeffersonianum*), an Endangered species in Canada, co-exist with unisexual, all-female, salamanders that use Jefferson Salamander sperm for recruitment. What happens when this sperm donor is lost in a population? It has been speculated that unisexual individuals might reproduce parthenogenetically (no sperm required) or that sperm can be obtained from other species. We sampled a salamander breeding pond in 2009, 2011, and 2015. Microsatellite analyses of 45 unisexuals captured in 2009 documented triploid *A. laterale*– (2) *jeffersonianum* (LJJ), tetraploid (LJJJ, LLJJ) and pentaploid (LJJJJ, LLJJJ) biotypes. Unisexuals declined to 21 individuals in 2015 comprising two biotypes (LJJ and LLJJ). Jefferson Salamanders, the expected sperm donor for unisexuals in this pond, were not found in any year but Spotted Salamanders (*Ambystoma maculatum*) were abundant. Unisexuals left the pond without laying eggs and proved negative when tested for the presence of sperm in their cloacae. Our study rejects hypotheses that unisexual salamanders in this population use Spotted Salamanders as a sperm donor, or that they reproduce parthenogenetically when acceptable sperm donors are not available. Without immigration or the introduction of a suitable sperm donor, this population is likely to become extirpated. The presence of unisexuals is used as an indicator that a suitable sperm-donating species is present, but our study demonstrates that this is not always the case. It is likely that there are more populations similar to the one that we sampled, suggesting that the Jefferson Salamander may be more imperiled than currently thought.**

Key Words.—conservation; drift fence; microsatellites; pitfall trap; reproduction; Visible Implant Elastomer (VIE) tags.

Introduction

Confirming the presence of individuals of rare species in a population can be difficult, but confirming the extirpation of a species from a population is much more challenging. The Jefferson Salamander (*Ambystoma jeffersonianum*) is listed as an Endangered Species in Canada (Committee on the Status of Endangered Wildlife in Canada [COSEWIC] 2010). The species has disappeared from many historic locations and the remaining locations are threatened by development, loss of habitat, habitat fragmentation, and possibly climate change (Jefferson Salamander Recovery Team 2010). Its habitat is protected by a specific regulatory amendment (Ontario Regulation 242/08) for the Jefferson Salamander that came into effect in 2010 under the Endangered Species Act, 2007 (ESA) of the Government of Ontario. The ESA habitat regulation of Jefferson Salamander also includes protection for Jefferson-dominated polyploids, which are unisexuals that contain two or more Jefferson Salamander genomes (e.g., LJJ and LJJJ biotypes). Currently, a 300-m radius around ponds used by Jefferson Salamander and associated unisexuals is protected. In addition, potential suitable breeding ponds within 1 km of a pond that is known to be used by these salamanders are protected along with a minimum 200-m wide corridor between the ponds. The protected habitat should allow for population expansion, immigration, and dispersal. Prior to 2010, planning authorities in Ontario were using a 30-m buffer around breeding ponds, and substantial foraging and overwintering habitat has been lost. In all Canadian populations, Jefferson salamanders co-exist with unisexual (female) *Ambystoma* and unisexuals are normally much more abundant (COSEWIC 2010). Surveys, especially those that rely on a random sample of individuals in a population, often document the existence of unisexual salamanders but not Jefferson Salamanders in sites within the range of Jefferson Salamanders (Bogart and Klemens 1997, 2008). Female Jefferson Salamanders are morphologically similar to the unisexuals they live with but the salamanders can be distinguished using genetic markers that are also used to identify the biotype and ploidy of unisexuals (Ramsden et al. 2006).

Mole Salamanders (genus *Ambystoma*) are seldom observed in their terrestrial habitat, but adults of most species migrate to ponds early in the spring and engage

in active, protracted breeding aggregations (Petranka 1998). Limited data (Schoop 1965; Douglas and Monroe 1981; Raymond and Hardy 1990) indicate that individuals continually return to the same pond to breed. Not surprisingly, population surveys and habitat protection for conservation purposes for these salamander species target breeding ponds. Based on an observation of a suspected Jefferson Salamander in Waterloo Region in southwestern Ontario from an area where Jefferson Salamanders had not previously been confirmed, potential Jefferson Salamander breeding ponds were surveyed using drift fences and pitfall traps as part of an Environmental Impact Study related to a proposed residential development.

Unisexual salamanders are mostly triploid (Bogart and Klemens 1997, 2008) and LJJ is the expected biotype in Jefferson Salamander breeding ponds. Unisexuals are expected to reproduce by gynogenesis so sperm is used only to initiate egg development (Elinson et al. 1992) and the offspring have the same genotype as their mother. Although ploidy elevation is relatively rare in most unisexual populations, tetraploid individuals have been found in various frequencies in several populations and the frequency of tetraploid offspring from triploid females can be increased by elevating the temperature of fertilized eggs (Bogart et al. 1989). In such cases, a sperm cell not only stimulates development of an egg, but is also incorporated. If a triploid LJJ female has tetraploid LJJJ offspring, the male sperm donor must be a Jefferson Salamander and if an LJJ female has tetraploid LLJJ offspring, the male sperm donor is a Blue-spotted Salamander (*A. laterale*). Pentaploids are very rare but have been encountered elsewhere (Lowcock and Murphy 1991). Pentaploid biotype LLJJJ could be derived from a LJJJ tetraploid egg that incorporated a Blue-spotted Salamander sperm cell (L genome) or from a LLJJ tetraploid that had a Jefferson Salamander sperm donor and incorporated a J genome. Blue-spotted Salamanders do not have a conservation status in Canada and are more common in this general area of Ontario than Jefferson Salamanders. Possibly, Jefferson Salamanders are extirpated from this population and the unisexual salamanders have more recently used male Blue-spotted Salamanders as sperm donors. LJJ and LJJJ unisexuals could produce gynogenetic offspring with the same biotypes as their mother irrespective of the sperm donor that is used.

We believe we are dealing with a closed population at this pond. Our study pond is isolated from other known breeding ponds and is at the western edge of the range of *Ambystoma jeffersonianum* in Ontario, Canada. Eight additional ponds within 1 km of the study pond have been sampled and none have been documented to support breeding members of the complex, although seven of them support Spotted Salamanders (*A. maculatum*). We conducted an additional more intensive study of the salamanders in this population in 2015 to test fundamental hypotheses with respect to recruitment and persistence of unisexual *Ambystoma* when a sperm donor is very rare or is extirpated. There are many similar populations over the range of unisexual *Ambystoma*. Specifically, we wished to know if sperm donors were present in the pond and if so, what species was being used by unisexual salamanders. We also wanted to know if there was recruitment of unisexual salamanders with or without a sperm donor. A more complete understanding of recruitment of unisexual *Ambystoma* could assist recovery efforts for the Jefferson Salamander in southern Ontario and elsewhere where unisexual salamanders interact with species at risk.

Materials and Methods

Surveys.—We carried out intensive, drift fence surveys in 2009 and 2015. In 2010 we captured one unisexual female, and in 2011 we caught 13 unisexual females from the pond using minnow traps. We took tissue samples for genotype identification. We released six individuals and kept seven in captivity to lay eggs in anticipation of obtaining ploidy-elevated larvae that could be used to confirm whether Jefferson Salamander or Blue-spotted Salamander males were used as sperm donors in the pond (Bogart and Bi 2013).

Drift fence.—We constructed drift fences with pitfall traps according to Dodd and Scott (1994). In 2009, we operated six drift fences 30 m in length each with three pitfall traps on either side of them, for a total of 36 traps. At this time, the drift fences did not completely surround the breeding pond. During the fall of 2014, we completely encircled the pond with drift fencing consisting of heavy-duty plastic silt fence stapled to stakes. The plastic was buried in the substrate to a depth of approximately 10 cm to prevent salamanders from burrowing under it. We installed pitfall traps (new, unused 3-L paint cans) at approximately 5 m intervals along the fence. We installed traps on both sides of the fence so that salamanders entering and leaving the pond could be captured. The total length of fence was approximately 810 m and we installed 191 pitfall traps (Fig. 2). We rolled down every other section of fence and sealed the traps to ensure wildlife movement was not disrupted between the time of the fence installation and the spring salamander migration.

We opened the traps on the outer side of the fence 1 April 2015; whereas, we opened the traps on the inner side of the fence 2 April 2015. Not every trap could be opened at that time because of thick ice; they were opened as soon as they were ice-free. We placed leaf detritus and small pieces of untreated sponge within

each trap to maintain moisture. We placed rocks on top of traps to deter predators but still allow amphibians to be captured. We measured snout-vent length (SVL) and marked all individuals captured in pitfall traps with as little handling as possible, and then released them on the opposite side of the fence where they were captured. We placed released amphibians under leaves or other cover to minimize the potential for predation. On 20 May 2015, we removed all fencing, pitfall traps, and other equipment from the site.

Marking.—We individually marked *Ambystoma laterale–jeffersonianum* complex salamanders, Spotted Salamanders, and Eastern Newts (*Notophthalmus viridescens*) with Visible Implant Elastomer (VIE) tags. It was anticipated that this study presented the opportunity to collect valuable data on the migration and dispersal of all these species. VIE is considered a humane and effective marking method for amphibians (Supsford et al. 2014). We followed the VIE color marking SalaMarker protocol of MacNeil et al. (2011). This protocol standardizes an alpha-numerical system for numbering salamanders and also provides a computer program for determining the sequence in which salamanders should be marked. Use of this program was essential to ensure that different fieldworkers could confidently assign unique tags to each salamander. The tags consisted of a small injection of bio-compatible colored elastomer (silicone) beneath the epidermis of the salamander. We used a hypodermic needle to inject liquid elastomer before it cured to a semi-solid state. We used fluorescent red, yellow, and orange colors. Because of the dark pigmentation of the skin of salamanders, we thought that even if tags were not visible to the naked eye, they would be detected with ultraviolet light. We used six specific locations for marking salamanders, including the front legs, the back legs, and both sides of the base of the tail. We marked each salamander on three locations with as many as three colors.

Examination for sperm cells.—In 2015, we examined *Ambystoma laterale–jeffersonianum* complex females captured in the pitfall traps while leaving the pond for the presence of sperm cells (Bogart and Licht 1987). We transported the salamanders to the laboratory in a clean container with a small amount of pond water and detritus on the day of capture. In the lab, we placed the salamanders in a bath of the anesthetic MS222 (1% tricaine methane sulfonate, buffered to pH 7.0 with NaOH). When they were relaxed and calm, we placed them upside down on a damp paper towel and rinsed their cloacal cavity with an amphibian saline solution using a glass pipette. We moved the pipette around the walls of the cloaca and placed a drop of cloacal fluid on a microscope slide and then applied a cover slip. We viewed slides with a phase contrast microscope using a 20 × objective. We rinsed individuals with tap water and kept them in their original containers until they fully recovered from the anesthetic. We returned females to the pond the same day (usually within a few hours) when they had visibly recovered from the anesthetic.

Microsatellites.—We excised tail tips (approximately 3 mm) from each salamander that we collected and that we identified visually as belonging to the *Ambystoma laterale–jeffersonianum* complex. We preserved the tail tips in 70% ethanol for DNA extraction and microsatellite analyses using the same methods and microsatellite loci that were used by Bogart et al. (2007, 2009) and also used to identify the unisexuals from this population in 2009 and 2011. In the present study, we added a sixth microsatellite DNA locus (AjeD75; Julian et al. 2003) using an annealing temperature of 58° C. We assigned unisexual biotype or genomotype (Lowcock 1994) according to the number of genomes that were present based on genome specific microsatellite DNA alleles (Ramsden et al. 2006). We made an effort to address possible scoring errors that might be mistaken for mutations. The primers for all loci that we examined amplify tetra-nucleotide repeats, which are easier to score than di- or tri-nucleotide repeats. Samples were all amplified more than once, and the positions of the samples on the gel were changed to confirm four base fragment size differences.

Results

Population size and migration.—We did not find either Jefferson or Blue-spotted salamanders during any of the pond surveys. Using microsatellite DNA markers, we identified 45 unisexual salamanders that represented four biotypes in this salamander complex from individuals collected from the pitfall traps at the only pond that was found to have Jefferson Salamander complex salamanders in 2009 (Fig. 1). They included triploid *Ambystoma laterale* – (2) *jeffersonianum* (LJJ), tetraploid *A. laterale* – (3) *jeffersonianum* (LJJJ), tetraploid A. (2) *laterale* – (2) *jeffersonianum* (LLJJ), and pentaploid A. (2) *laterale* – (3) *jeffersonianum* (LLJJJ) biotypes. We also captured 77 Spotted Salamanders (*A. maculatum*).

We collected only 21 unisexuals in 2015 when the pond was completely encircled with drift fences, but we made 559 captures of 302 individual Spotted Salamanders, including 135 males. In 2015, migration to the pond extended over two weeks (3–17 April) and the time that a unisexual remained in the pond ranged from 3–24 d with a mean of 10.06 ± (SD) 5.66 d. We do not know the pond residency for a few individuals that were not captured entering as well as leaving the pond.

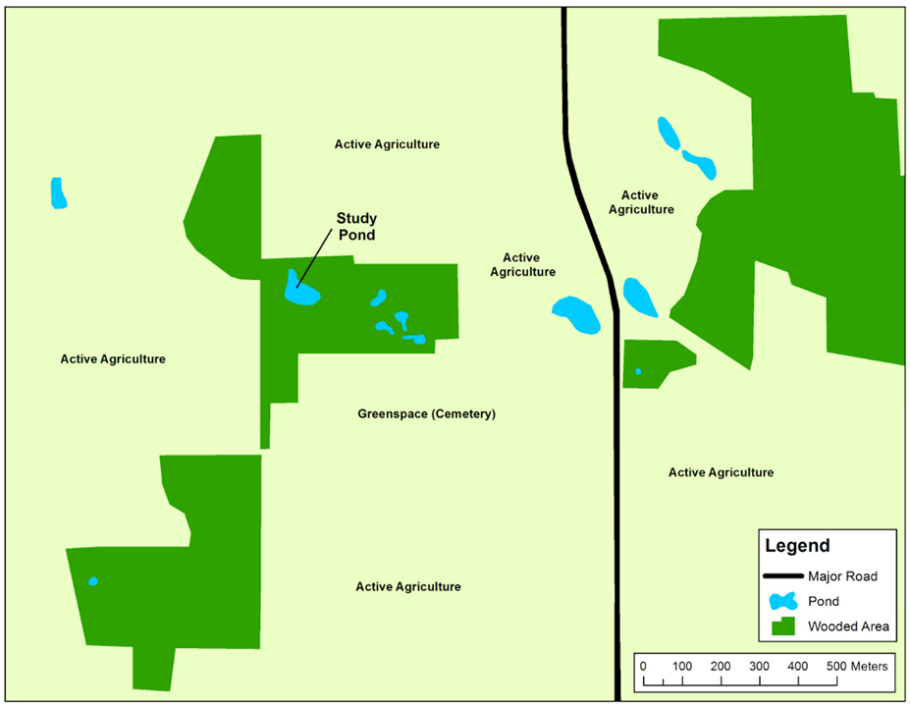

Figure 1. Landscape in which the study pond exists is characterized by a network of rural farm properties, active agricultural lands (row crops) and isolated woodlands (green). Multi-year minnow trap and egg mass surveys were performed at all of the potential salamander breeding ponds (blue). Individuals of the Jefferson Salamander complex (*Ambystoma*) were only found in one pond (study pond).

Five individual unisexuals entered the pond more than one time but none of the Spotted Salamanders visited the pond more than once (Table 1). The time Spotted Salamander males spent in the pond ranged from three to 39 d with a mean of 12.33 ± 7.8 d. Female Spotted Salamanders spent one to 27 d in the pond with a mean of 6.49 ± 4.3 d (Fig. 3).

Sperm cells.—Two of the seven unisexual females we captured in 2011 laid 21 and 29 eggs in the laboratory that did not develop. We returned the salamanders to the pond after 2 mo in captivity. We examined 12 unisexual salamanders that we captured leaving the breeding pond in 2015 for the presence of sperm cells (Table 1). Even though female 43941 was in the pond for 16 d and female 43943 was in the pond for 13 d, none of the 12 salamanders tested positive for the presence of sperm. The females were obviously gravid when we tested them. Two Spotted Salamanders tested positive for the presence of sperm cells.

Microsatellites.—We identified salamander biotypes and ploidy based on genome specific microsatellite DNA alleles and the number of fragments observed at any of the six loci (Table 2). We found 43 unique multi-locus genotypes (MLGs) in the 79 unisexual salamanders sampled (Appendix). In many cases, the only difference between MLGs was a four-base change in a fragment at one of the six microsatellite loci or a loss of a microsatellite. The triploids can be grouped into three major clones (G1 to G11; G12 to G20; G21 to G32) if a loss of a fragment or a single four-base shift is accepted as a consequence of expected microsatellite mutation (see Discussion). The tetraploids and pentaploids have one (tetraploid) or two (pentaploid) additional genomes but have obvious affinities to the major clones. Six unisexuals sampled in 2009 (G7) had the same MLG and six individual unisexuals sampled in 2011 (G12) share a MLG. The same two MLGs (G4; G6) were found in individuals sampled in 2009 and 2011. The 21 unisexuals sampled in 2015 represent 15 different MLG clones, none of which were found in 2009, 2010 or 2011.

Salamander size.—Unisexual salamanders that we collected in 2015 (n = 21) had a mean SVL of 8.7 cm ± 0.5 (range, 7.5–9.5). We also measured unisexuals from a population of Jefferson Salamander–dependant unisexuals on the Niagara Escarpment in Ontario, Canada, where Jefferson Salamanders are relatively numerous (COSEWIC 2010, location A). Unisexuals in that population (n = 336) had a mean SVL of 8.1 cm ± 0.6 (range, 5.6–9.5; Fig. 4). The largest individual in both populations had a SVL of 9.5 cm. Mean SVL

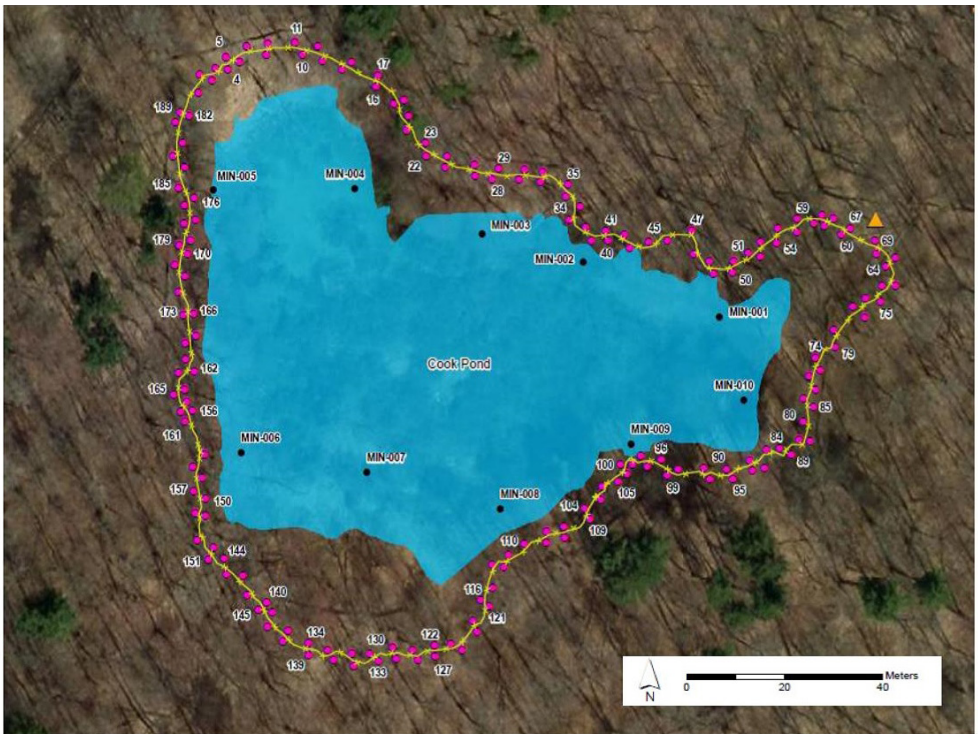

Figure 2. Salamander (*Ambystoma*) breeding pond study site in southwestern Ontario. The pond was completely surrounded with a drift fence (yellow) and 191 pitfall traps (pink) situated on both sides of the fence. Ten minnow traps (black, MIN) were set in the pond in an attempt to catch females prior to egg deposition. The orange triangle is the location of the field work station.

of unisexual salamanders in the two populations were significantly different (t = -4.17, df = 355, P < 0.001).

Discussion

***Reproduction of unisexual* Ambystoma**.— Gynogenesis can be defined as sperm-dependant parthenogenesis. Sperm cells only serve to activate egg development and the genome of the male is not incorporated in the zygote. Macgregor and Uzzell (1964) and later Cuellar (1976) found that triploid unisexuals of the *Ambystoma jeffersonianum–laterale* complex produced triploid eggs by premeiotic endomitosis. This finding provides the mechanism for triploid females to produce triploid offspring but is not, however, direct evidence for gynogenesis although it was so interpreted by Macgregor and Uzzell (1964). Premeiotic endomitosis is a common meiotic mechanism used by asexual organisms and has been documented to occur in several asexual invertebrates and vertebrates, such as parthenogenetic grasshoppers (*Warramaba virgo*; White et al. 1963) and parthenogenetic diploid (*Aspidoscelis tesselatus*) or triploid (*A. exsanguis*) lizards (Lutes et al. 2010). Cuellar (1976), in his cytological examination of unisexual LLJ eggs, failed to detect sperm in any oocyte and suggested that the sperm may have invaded the oocyte just prior to ovoposition, or the sperm was lost in the discarded jelly capsule, or that females of these populations did not require males and were truly telytokous. Finding only unisexuals, or triploids, and no diploid members of the Jefferson Salamander complex in Delaware and Boone Counties in Indiana led Uzzell (1969) to conclude that these triploids appeared to have escaped not only the environmental hazards, but also the dependence of males of the complex for stimulation of their eggs to develop. In an ecological study of *Ambystoma laterale* and unisexual LLJ, Wilbur (1971) speculated that the present gynogenetic relationship is probably a transitory stage in an evolutionary sequence that could result in the reproductive independence of the triploid line by the evolution of a parthenogenetic or of a bisexual, tetraploid form. Downs (1978) examined unisexual *Ambystoma* that included genomes of *A. laterale* and *A. texanum* (Small-mouthed Salamander) on the Bass Islands in Lake Erie and, supported by the apparent absence of males on North Bass Island, concluded that the island *Ambystoma* were capable of thelytokous parthenogenesis. Kraus (1985) agreed with Downs for the *A. laterale–texanum* unisexuals on North Bass Island and tentatively considered parthenogenesis to be the most likely form of reproduction in this taxon.

Not finding expected males is weak evidence for parthenogenesis. Indeed, Bogart (2003; figure 3.3) showed a sperm cell that was retrieved from the cloaca of a North Bass Island *A. laterale* – (2) *texanum* (LTT) unisexual. The male that provided the sperm cell is

unknown but was a North Bass Island dweller. Pelee Island, in Lake Erie, also has unisexual biotypes with various combinations of *A. laterale* and *A. texanum* genomes as well as bisexual *A. laterale* and *A. texanum* (Bogart et al. 1985). In the only previous field study that tested the requirement of sperm for egg development, Bogart and Licht (1986) examined 61 unisexual females that were egg-producing from Pelee Island for the presence of sperm in their cloacae. Most of the eggs from 23 sperm-negative females did not develop at all and none of those females produced eggs that hatched. Only sperm-positive females produced viable progeny and most of these females produced offspring having the same ploidy as the female as well as ploidy-elevated offspring. Two laboratory experiments artificially inseminated unisexual eggs with sperm derived from known males. Morris and Brandon (1984) used eggs from unisexuals, which they identified morphologically as *A. platineum* (= LJJ), and applied sperm from *A. texanum* and *A. maculatum*. Control eggs (no sperm) did not develop. Four of 97 eggs in the cross with *A. texanum* sperm hatched but none of the 117 eggs used in the cross with *A. maculatum* hatched although several eggs did develop and one reached the tail-bud stage. Bogart et al. (1989) used eggs from triploid LJJ females and sperm from *A. tigrinum* (Eastern Tiger Salamander), *A. laterale*, and *A. maculatum*. All these crosses gave rise to transformed juveniles and it was determined that temperature played a role in the frequency of ploidy-elevated offspring. More tetraploid offspring were produced at a higher temperature (15° C) in crosses with *A. tigrinum* and *A. laterale*. Offspring in the crosses with *A. maculatum* were only produced at 6° C.

Table 1. Unisexual Salamander migration to and from the pond in 2015. Female 43938 migrated to the breeding pond three times and was not captured leaving the pond on her last visit. The total days in the pond includes more than one visit for some females. The Catalogue Number (Cat. No.) is that of J. P. Bogart. Numbers with an asterisk (*) were females that we examined for the presence of sperm. Visible Implant Elastomer (VIE) tag colors were Red (R), Yellow (Y), and Orange (O). Tags were injected into the dermis at three of six selected locations.

Cat. No.	VIE Tag	To pond	Trap #	From pond	Trap #	Days in pond
43935*	R1R2R4	3 April	067	17 April	080	15
43936	R1R2R3	3 April	121	20 April	098	17
43937	R1R2R5	3 April	151	10 April	174	7
43938	R1R2R6	3 April	159	8 April	168	5
		9 April	185	10 April	020	1
		14 April	007	?	?	? (Total > 6)
43939	R1R3R4	3 April	179	10 April	004	7
43940*	R1R3R5	3 April	179	14 April	002	11
43941*	R1R3R6	3 April	11	8 April	170	5
		10 April	185	20 April	164	11 (Total 16)
43942	R1R4R5	3 April	037	?	?	?
43943*	R1R4R6	3 April	043	10 April	166	7
		14 April	179	20 April	178	6 (Total 13)
43944*	R1R5R6	5 April	135	10 April	160	5
				14 April		3 (Total 8)
43946	R2R3R5	8 April	121	20 April	98	3
43945	R2R3R4	?	MIN-004	?	?	?
43947*	R2R3R6	9 April	127	17 April	18	8
43948	R2R4R5	9 April	185	?	?	?
43949*	R2R4R6	10 April	105	20 April	178	10
43950*	R2R5R6	?	?	20 April	100	?
43951*	R3R4R5	14 April	179	17 April	12	3
				20 April	6	? (Total > 3)
43953*	R3R5R6	?	?	17 April	184	?
43955	Y1Y2Y3	17 April	157	11 May	146	24
43954*	R4R5R6	?	?	17 April	178	?
43956*	O1O2O3	?	?	20 April	10	?

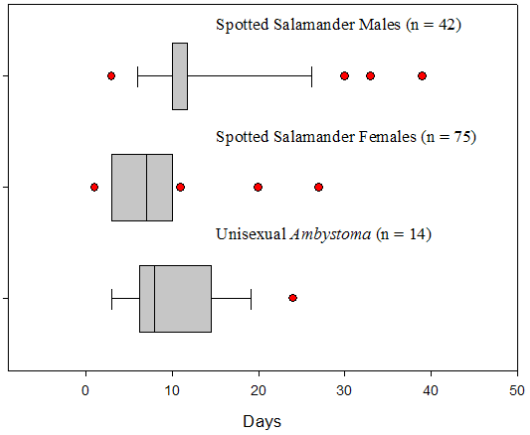

Figure 3. Time of pond residency for Spotted Salamanders and unisexual salamanders (*Ambystoma*). The shaded box boundaries are 25 and 75%. The whiskers represent 10 and 90% boundaries. The black line represents the mean number of days individuals spent in the pond. The red dots represent outlier individuals.

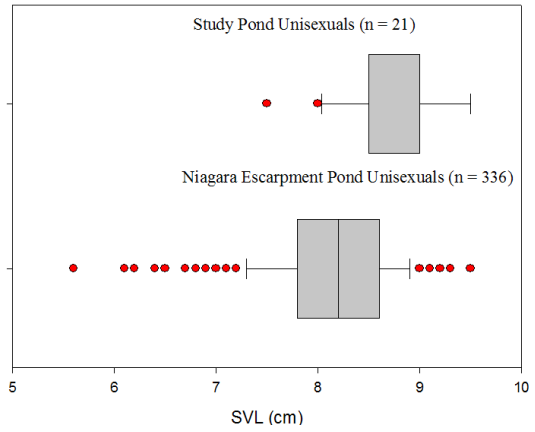

Figure 4. Size comparison (SVL) of Jefferson Salamander–dependant unisexuals (*Ambystoma*) from our study population where sperm donors were not found (top) and a population that contains Jefferson Salamander sperm donors (bottom).

The reproductive mode of unisexual *Ambystoma* was described as being kleptogenetic, which is a unique reproductive system (Bogart et al. 2007). Unisexuals represent a monophyletic lineage that has persisted for approximately 5 million y (Bi and Bogart 2010) by stealing sperm from normally bisexually reproducing males. If sperm cells serve only to initiate egg development (gynogenesis) the offspring have the same genotype as their mother, but the sperm is incorporated in some eggs, which adds a genome and increases the ploidy level in offspring. Genome replacement, where a genome in a unisexual individual is exchanged for a sperm-derived genome has also been documented (Bi et al. 2008). Ploidy elevation and genome replacement provide genetic variation in unisexual populations as do chromosomal mutations, such as intergenomic translocations (Bi and Bogart 2006; Bi et al. 2007). The success and persistence of unisexual *Ambystoma* over their extensive range in eastern North America can be attributed to kleptogenesis.

Table 2. Triploid (3n) LJJ, tetraploid (4n) LJJJ and LLJJ, and pentaploid (5n) LLJJJ unisexual Ambystoma biotypes that were identified using microsatellite DNA alleles in a southwestern Ontario pond during three time periods. Nuclear genomes (chromosome complements) include *A. laterale* (L = haploid set of chromosomes) and *A. jeffersonianum* (J = haploid set of chromosomes). Drift fences and pitfall traps were used to capture salamanders in 2009 and 2015. Only a few minnow traps were used to capture salamanders in 2010 and 2011.

Year	Total n	LJJ (3n)	LJJJ (4n)	LLJJ (4n)	LLJJJ (5n)
2009	45	36	3	3	3
2010	1	1			
2011	13	12	1		
2015	21	20		1	

Acceptable sperm donors.—So far, five bisexual species (*A. barbouri*, *A. jeffersonianum*, *A. laterale*, *A. texanum*, *A. tigrinum*) are known sperm donors for unisexual *Ambystoma* (Bogart et al. 2009). All unisexuals have at least one *A. laterale* chromosome complement (genome) and one or more genomes of these other species. The 45 unisexuals and 77 Spotted Salamanders collected in 2009 was likely an underestimate of the population size at that time because the pond was not completely surrounded with drift fences. This represents at least a 50% unisexual population decline in 6 y; in contrast, the number of captures of Spotted Salamanders increased more than seven-fold between 2009 and 2015. We could not confirm that either *A. jeffersonianum* or *A. laterale* were present in the breeding pond in 2009, 2010, 2011, or 2015. The large number of Spotted Salamander males in the study pond in 2015 (135) provided a test for the hypothesis that male Spotted Salamanders could be used as an additional sperm donor for unisexual recruitment in nature. Bogart et al. (1989) demonstrated that it is possible for Spotted Salamander sperm to be used for recruitment of unisexual gynogenetic offspring and the resulting offspring would not provide any genetic evidence that would identify the male that was used. Spotted Salamander males were abundant in the breeding pond but female unisexuals left the breeding pond without depositing eggs and tested negative for the presence of sperm in their cloacae. Thus, we reject the hypothesis that Spotted Salamander sperm is used to induce gynogenetic development of unisexual eggs in this population.

Selander (1994) also tested the hypothesis that Spotted Salamanders could serve as sperm donors in a population of unisexuals in a pond in Ohio. His spring pond census included 2,617 unisexual salamanders

with biotypes that included genomes of *A. laterale*, *A. jeffersonianum*, *A. texanum*, and *A. tigrinum*. In addition to these unisexuals, there were 2,861 Spotted Salamanders, 537 Tiger Salamanders, and a single pair of Small-mouthed Salamanders (one male and one female). In his mating experiments, unisexuals paired with Tiger Salamander males successfully produced fertile eggs but none of the 36 unisexuals that he paired with Spotted Salamander males produced eggs that developed. This would be consistent with not finding sperm cells in the unisexuals that we examined, which left the breeding pond without laying eggs. The two salamanders that tested positive for the presence of sperm in our study were male and female melanistic (non-spotted) Spotted Salamanders that were initially suspected of being a male Jefferson Salamander and a unisexual. Microsatellite analyses confirmed that the tested individuals were Spotted Salamanders. Spotted Salamanders have unique microsatellite fragment sizes for some of the loci we examined (AjeD75, AjeD346, AjeD422) and microsatellites are not amplified for locus AjeD378 (data not shown).

Pond residency.—The time that a salamander stays in a breeding pond may be related to environmental conditions and sex. Migration to and from a breeding pond normally coincides with rainy nights when the temperature is moderate (Downs 1989) but migration may not be strictly tied to a rain event (Brodman 1995). A longer residency for a male would provide time to court and mate with multiple females. Once mated, a female is expected to remain in the pond only for the time taken to lay eggs but her departure may be delayed if weather conditions are unsuitable. We compared the residency of Spotted Salamanders to the unisexuals in our studied pond because we wanted to confirm the presence of male Spotted Salamanders during the time that unisexuals were also in the pond and could possibly be used as sperm donors. On average, unisexuals stayed in the pond longer than Spotted Salamander females and, unlike any Spotted Salamander female, some unisexuals left the pond, and subsequently returned. Because acceptable sperm donors are often rare in unisexual breeding ponds, this strategy may be important. Some sperm donors might enter the breeding pond later in the season or have time to recover from an active time of breeding to produce additional spermatophores. If most females, including Jefferson Salamander females, in a breeding pond have bred, unisexuals would be expected to have less competition for available males at different time periods during the season.

Four individuals that we marked going to the pond were not recovered leaving the pond, which is probably related to environmental conditions. In southern Ontario, May 2015 was hotter and drier than average with only 5.2 mm of rain falling between the first of the month and when the traps were closed on 20 May, the majority (4.4 mm) of which fell during a 4-d period. Of 123 Spotted Salamanders we recorded leaving the pond, only seven individuals left during the month of May, all during the wetter 4-d period. All other Spotted Salamanders and unisexuals were captured leaving the pond during the month of April. After the traps were closed, conditions remained very dry until 30 and 31 May when there were large precipitation events (36 and 24 mm respectively). We hypothesize that the remaining individuals in the pond waited for these rain events to leave or they were preyed upon before they reached the fence.

It is more difficult to explain why some individuals captured in minnow traps or captured leaving the pond were not marked when they entered the pond. Possible explanations include a breach of the drift fence during a storm event that knocked part of the fence down in a high migration area, salamanders might have been situated closer to the pond than was the drift fence at the beginning of the season, and some VIE tags may have been lost or misread. We confirmed pond residency for 14 unisexual individuals from entering and exiting dates.

Microsatellites.—Identification of genomotypes of unisexual salamanders and their bisexual sperm donors is accomplished using DNA that is extracted from small tissue samples and used as a template for specific primers that amplify highly variable short tandem repeats (microsatellites) in the nuclear genome. Multi-locus genotypes (MLGs) from a relatively few loci are widely used in forensics to identify individuals and their relatives. Bisexually reproducing individuals normally have unique MLGs. Gynogenetic offspring of unisexual *Ambystoma* are expected to have the same MLG as their mother. Thus, if a population of unisexuals was founded by a single unisexual female that reproduced only by gynogenesis, the resulting unisexual lineage in that pond should be genetically identical as all individuals would be members of the same clone. We observed 43 unique MLGs in 79 unisexual individuals (see Appendix). The ratio of #MLG/N is also termed the G:N ratio or the genetic diversity index and can be used to estimate the proportion of identical (clonal) genotypes in a population. The G:N ratio ranges from 1/N, where all individuals have the same MLG and are members of a single clone, to 1.0, where all individuals have a unique MLG. Normally, in microsatellite investigations, the G:N ratio increases with the number of loci that are used and bisexually reproducing individuals have a G:N ratio of 1.0. The calculated G:N ratio for unisexuals in our study pond is 0.54 which compares with 0.83 that was calculated for unisexuals in another southern Ontario Jefferson Salamander pond studied by Ramsden (2008)

where the sperm donor (*A. jeffersonianum*) exists and 0.93 in a pond where unisexuals breed in the same pond with both *A. jeffersonianum* and *A. laterale* (Bogart et al. 2007). In southern Québec, one pond was found to have only LJ unisexuals (n = 36) and Spotted Salamanders (Noël et al. 2011): the unisexual G:N ratio was 0.14.

Our data reveal several MLGs that are differentiated by one or very few four-base changes in fragment sizes or a deletion. Microsatellites are known to have a high rate of mutation and their evolution is a complex process that involves increases and decreases of repetitive units by DNA slippage and can even be induced by DNA polymerase that is used in polymerase chain reaction (PCR) amplification (Ellegren 2004). The mutation rate for microsatellites in unisexuals is unknown but Bulut et al. (2009) calculated a microsatellite mutation rate of 4.98 x 10-3 for one tetranucleotide microsatellite locus in *A. tigrinum* based on unexpected 4-base changes observed in 10 of 1005 offspring from the same parents. There were no observed mutations at four other microsatellite loci. The mutation rate was calculated as the number of observed mutations / 2x the number of offspring sampled in this diploid species. Using that calculation, triploid (3x) and tetraploid (4x) offspring should have lower mutation rates. Over time, and depending on the microsatellite loci examined, the number of unisexual MLGs might increase by mutations if there is no recruitment but it is very surprising that none of the unisexuals we sampled in 2015 had the same MLG as salamanders sampled in 2009, 2010 or 2011. Salamanders were not individually marked in the previous years but salamanders collected in 2015 are unlikely to be recent immigrants because there are no breeding ponds in the vicinity and the woodlot where the pond is found is surrounded by agricultural fields and urban development. That few unisexuals share the same MLG supports our contention that unisexuals have not successfully bred in this population for a long period of time.

The Clanton Effect.—Clanton (1934) was the first investigator to recognize the existence of unisexual *Ambystoma*. His breeding experiments clearly showed that Light Individuals were all females and when mated with Dark males only produced Light females, while Dark Individuals had a 1:1 sex ratio and Dark females produced male and female Dark individuals. From his observations in southern Michigan ponds, he hypothesized that populations of unisexuals would increase and out-compete females of the bisexual species for available spermatophores. The logical outcome, according to Clanton, would be a population crash of bisexuals that would be followed by a population crash of unisexuals. This phenomenon was coined The Clanton Effect by Minton (1954). It is possible that the loss of Jefferson Salamanders, and possibly Blue-spotted Salamanders in the pond that we studied can be explained by the Clanton Effect, but unisexual salamanders have co-existed with their sperm donors for a very long period of time and have likely evolved an evolutionarily stable strategy (ESS; Maynard Smith 1982) that would counter extirpation. Male ambystomatids produce many more spermatophores than would be required to sustain the bisexual population and they produce twice as many spermatophores when they are courting conspecific females than they do when they court unisexuals (Uzzell 1969). Dawley and Dawley (1986) showed that Jefferson Salamander males can distinguish between conspecific females and unisexuals. In their experiments, Jefferson Salamander males preferred conspecific females.

Implications for conservation.—The main factors contributing to the decline of *Ambystoma jeffersonianum* in Ontario are habitat loss and alteration. Road mortality has also been observed to be a problem in habitats where breeding ponds and terrestrial habitats are transected by roads. The landscape in which the study pond exists is characterized by a network of rural farm properties, active agricultural lands (row crops) and isolated woodlands. A provincially significant wetland complex, consisting of a network of marshes and swamps, is also scattered across the landscape. Urban development is an increasing pressure in the general area, although in the immediate vicinity of the pond (up to about 1 km or more in each direction) the main anthropogenic activity has been agriculture. The portion of the woodland surrounding the pond where hibernation and foraging occurs has been left relatively undisturbed and the population of *A. maculatum* seems to be thriving. The forest is intact and native flora is abundant. The pond does receive surface water runoff from agricultural lands and water quality testing has shown increased levels of some contaminants; however, research has shown that water chemistry and water quality parameters are not good predictors for the use of breeding ponds by the species (Bériault 2005). Four other small ponds occur in the same woodlot to the east of the study pond, which have also been sampled over 5 y for salamanders. This portion of the woodlot (under different landownership) has been disturbed as a result of selective logging and refuse disposal. One of the ponds also receives storm water inputs from the adjacent cemetery that has degraded water quality mostly related to elevated chloride levels. All four of these ponds support populations of *A. maculatum*.

Based on historical air photos and discussions with the current landowners, the extent and intensity of farming occurring around the study pond have not changed in at least 100 y. There have been no other

apparent disturbances to the study pond: no fish have been introduced; no major contaminants have been dumped; migration corridors have not changed; winter/foraging habitat has not been significantly logged or otherwise disturbed. From our investigation, the endangered Jefferson Salamander is extirpated from the study pond and has probably not bred in the pond for several years. We know that they bred in the pond historically because Jefferson Salamander–dependant unisexuals still exist and do not use available male Spotted Salamanders as sperm donors. The numbers of unisexuals in the pond have declined more than 50% in 6 y and they likely have a bleak future. It is tempting to blame the unisexual salamanders for the demise of the Jefferson Salamanders but they successfully co-exist in suitable habitat over the range of Jefferson Salamanders in Canada. The costs and benefits of the bisexual/unisexual relationship have not been adequately studied because few population-level studies have been performed and it is difficult to distinguish between bisexual and unisexual larvae, juveniles, or adults. The Clanton Effect may explain the loss of Jefferson Salamanders when a bisexual/unisexual ESS becomes unstable. As hypothesized by Minton (1954), new Jefferson Salamander immigrants would be required to recover stability. We believe that fragmentation and isolation of this population has arrested immigration. Spotted Salamanders co-occur with, and have similar life histories and habitat preferences to Jefferson Salamanders. The comparatively large population of Spotted Salamanders indicates that the habitat has probably not deteriorated for Jefferson Salamanders. If the Jefferson Salamander no longer exists, or breeds in a pond, unisexual salamanders may continue to migrate to the pond for several years with no chance for recruitment. If the population of unisexual salamanders is old, this could provide evidence for the extirpation of a sperm donor. If SVL can be used as a surrogate for age, it may be possible to predict the fate of Jefferson Salamanders in a population by monitoring recruitment and size of unisexuals. The small SVL outliers from the Niagara Escarpment population likely represent first-breeding individuals, providing evidence for recruitment in that population. It has traditionally been assumed that the presence of unisexuals is a positive indicator that an appropriate sperm-donating species is also present in the population. Our study demonstrates that this is not always the case.

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James (Jim) Bogart (left) got his Bachelor's degree from the University of Toronto, Canada, and went to the University of Texas in Austin for his M.A. (1967) and Ph.D. (1969). He was an Assistant Professor in the Department of Zoology at Louisiana Tech University in Ruston for five years. In 1975 he took a position in the Department of Zoology at the University of Guelph, Ontario, where he taught Herpetology and Comparative Anatomy until 2005. He continues his research as Professor Emeritus in the Department of Integrative Biology at the University of Guelph. His research focus is on polyploid amphibians and especially unisexual salamanders in the genus *Ambystoma*. **Jessica Linton** (middle) is an Environmental Consultant based in Waterloo, Ontario. In 2006, she completed an undergraduate degree in Environment and Resource Studies at the University of Waterloo and shortly after began working for her current employer, Natural Resource Solutions, Inc. In 2010 she obtained a Master's of Environmental Studies degree from the University of Waterloo. As a Consulting Biologist, Jessica has experience conducting a wide range of wildlife studies but she has particular interest and considerable field experience in designing and carrying out studies on herpetofaunal species at risk. Over the past 10 y, she has worked on a variety of projects focused on surveying and identifying habitat for ambystomid salamanders and turtle species at risk. Jessica also has particular expertise in butterfly ecology. She is a member of the arthropod specialist subcommittee of the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) and is the Editor of *Ontario Insects* and the *Ontario Lepidoptera Summary*. **Al Sandilands** (right) is an Environmental Consultant who works predominantly with species at risk. He obtained a B.Sc. from the University of Waterloo in 1970 and worked for a variety of consulting companies prior to establishing his own firm of Gray Owl Environmental Inc. in 2003. He has worked on more than 50 species at risk including completing inventories, status reports, research studies, a draft recovery strategy, and obtaining permits under the Ontario Endangered Species Act, 2007. In addition to amphibians and reptiles, he has a strong interest in birds. He is the author of a two-volume book on the habitat requirements, limiting factors, and status of the nonpasserine birds of Ontario. (Photographed by Jessica Linton).

Appendix. Multi-locus Genotypes (MLGs) and clones (G) for six microsatellite loci observed in 79 unisexual Ambystoma sampled from a southwestern Ontario pond in 2009, 2011, and 2015 when no sperm donor was collected. Microsatellite fragment sizes that are only found in A. laterale (L) (**bold**) or only found in *A. jeffersonianum* (J) (underline) were used to identify triploid, tetraploid, and pentaploid unisexual biotypes (Geno). Fragment sizes that are not marked can be found in both diploid species. Ploidy is determined by the number of genome specific fragments for all loci. For example, G33 is a pentaploid LLJJJ because there are three J fragments for *Aje*D346 and *Aje*D378 and two L fragments for *Aje*D94

					Microsatellite Locus					
G	n[1]	Year	JPB#[2]	Geno	*Aje*D75	*Aje*D94	*Aje*D283	*Aje*D346	*Aje*D378	*Aje*D422
1	1	2009	39888	LJJ	**128**/160/188	**150**/182/222	134/146/158	172/176/**288**	232/260	252/256/304
2	2	2009	39884	LJJ	**128**/160/188	**150**/182/226	134/146/158	172/176/**288**	232/260	252/256/304
3	2	2009	39889	LJJ	**128**/160/188	**150**/182/234	134/146/154	168/176/**300**	232/252	252/256/304
4	3	2009(2) 2011(1)	39938	LJJ	**128**/160/188	**150**/182/246	134/146/158	164/176/**288**	232/252	252/256/304
5	1	2015	43947	LJJ	**128**/160/188	**150**/182/246	134/146/158	164/176/**288**	236/256	252/256/304
6	7	2009(6) 2011(1)	39878	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	232/256	252/256/304
7	10	2009	39875	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	232/256	256/304
8	2	2011	41267	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	236/256	256/304
9	1	2015	43951	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	236/256	252/256/304
10	4	2015	43937	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	236/260	252/256/304
11	3	2015	43944	LJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	236/260	256/304
12	6	2011	41223	LJJ	**128**/176/188	**150**/202/210	146/154	180/184/**272**	260/264	232/240/256
13	1	2009	39907	LJJ	**128**/176/188	**150**/202/214	146/154	180/184/**272**	260/264	232/240/256
14	3	2015	43945	LJJ	**128**/176/188	**150**/202/214	146/154	180/184/**272**	264/268	232/240/256
15	1	2015	43949	LJJ	**128**/176/188	**150**/202/214	146/154	180/184/**272**	264/268	232/256
16	2	2009	39935	LJJ	**128**/176/188	**146**/202/214	146/154	180/184/**272**	256/264	232/240/256
17	4	2009	39883	LJJ	**128**/176/188	**146**/202/214	146/154	180/184/**272**	260/264	232/240/256
18	1	2011	41271	LJJ	**128**/176/188	**150**/202/210	146/154	180/184/**272**	260/264	232/240/256
19	1	2009	39887	LJJ	**128**/176/188	**150**/202/214	146/154	180/184/**272**	260	232/240/256
20	1	2015	43939	LJJ	**128**/176/188	**150**/202/210	146/154	180/184/**272**	264/268	232/240/256
21	1	2011	41224	LJJ	**132**/156/188	**150**/186/214	134/150	176/184/**304**	240/244	232/252
22	1	2015	43946	LJJ	**132**/156/188	**150**/186/214	134/146/154	176/184/**308**	244/248	232/248
23	2	2015	43935	LJJ	**132**/156/188	**150**/186/218	134/146/154	176/184/**308**	244/248	236/252/260
24	1	2009	39882	LJJ	**132**/156/188	**150**/186/218	134/150/154	176/184/**308**	240/244	232/252
25	1	2009	39904	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**304**	240/244	232/252
26	1	2015	43948	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**304**	244/248	232/248
27	1	2009	39891	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**308**	240/244	232/252

APPENDIX—CONTINUED.

G	n[1]	Year	JPB#[2]	Geno	Microsatellite Locus					
					*Aje*D75	*Aje*D94	*Aje*D283	*Aje*D346	*Aje*D378	*Aje*D422
28	1	2010	40488	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**308**	240/244	232/256
29	1	2015	43953	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**308**	244/248	232/248
30	1	2009	39903	LJJ	**132**/156/188	**150**/186/218	134/150	176/184/**308**	244/264	232/248
31	1	2009	39901	LJJ	**132**/160/184	**150**/186/218	134/150	176/184/**308**	240/244	232/252
32	1	2015	43942	LJJ	**132**/160/184	**150**/186/218	134/150	176/184/**304**	244/248	232/248
33	1	2009	39939	LLJJJ	**128**/160/188	**142**/**150**/182/226	134/146/154/158	168/172/176/**288**	232/256/260	252/256/304
34	1	2009	39940	LLJJ	**128**/160/188	**142**/**150**/186/234	134/146/154	168/176/**300**	232/256	252/256/304
35	1	2009	39880	LJJJ	**128**/160/188	**150**/182/210/218	134/146/150/154	172/176/180/**288**	232/252/260	252/256/304
36	1	2009	39937	LJJJ	**128**/160/188	**150**/186/234	134/146/154	168/176/**300**	232/252/256	252/256/308/**312**
37	1	2015	43943	LLJJ	**128**/160/188	**150**/186/234	134/150/158	168/176/**296**/**340**	236/260	256/308
38	1	2011	41268	LJJJ	**128**/176/188	**146**/190/202/214	146/154	172/180/184/**272**	256/260/264	232/240/244/256
39	1	2009	39899	LJJJ	**128**/176/188	**146**/190/202/214	146/154	172/180/184/**272**	260/264	232/240/244/256
40	1	2009	39945	LJJJ	**128**/176/188	**146**/202/214	146/154	180/184/**272**	256/264	232/240/256/308
41	1	2009	39876	LLJJJ	**132**/156/188	**142**/**150**/186/210/218	134/146/150/154	168/176/184/**308**	240/244/252	232/248/252/304
42	1	2009	39906	LLJJJ	**132**/156/188	**142**/**150**/186/218	134/150	176/184/**308**	232/244/256	232/252/264
43	1	2009	39900	LLJJ	**132**/160/184	**142**/**150**/186/218	134/150	176/184/**304**	260/264	232/248/260

[1]n is the number of individuals that have the same MLG for all six microsatellite DNA loci and are considered to be members of the same clone.
[2]JPB# is the catalogue number of J.P. Bogart for tissue samples that were collected from captured individuals and used for DNA extraction and microsatellite analyses. When more than one individual has the same MLG, a single JPB# is representative of that clone