THE INFLUENCE OF INTERSPECIFIC HYBRIDIZATION ON THE MORPHOLOGY OF THE SPERMATOOZOA OF *AMBYSTOMA* (CAUDATA, AMBYSTOMATIDAE)

R. A. BRANDON, J. MARTAN, J. W. E. WORTHAM
AND D. C. ENGLERT

*Department of Zoology, Southern Illinois University,
Carbondale, Illinois 62901, U.S.A.*

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Summary. Spermatozoa were taken from the ductus deferens and freshly deposited spermatophores of four closely related species (*t*, *d*, *z*, *m*) of *Ambystoma* and eight interspecific hybrids among them. After the spermatozoa had been spot-smeared and stained, the lengths of the head, neck, and whole spermatozoon were measured. Multiple comparisons among means of the measurements revealed significant differences in (1) lengths of the head with acrosome among all four parental species; (2) lengths of the neck among all but *t* versus *d*; and (3) length of the whole spermatozoon between *t* and the other three species, and between *z* and *m*. The measurements were greatest for the spermatozoa of *A. tigrinum*: whole spermatozoon, 497 μm ; head with acrosome, 125 μm ; and neck, 13.8 μm . In every case but one (*d/m*), mean measurements of hybrid heads with acrosomes lay between those of the parental species. Paternal influence was clear in reciprocal crosses between *m* and *z*. In every case but two (*m* $\times$ *t/d*; *d/z*), mean measurements of hybrid necks lay between those of the parental species. In total sperm length, the *m/t* hybrid lay between the parental species.

INTRODUCTION

The genetic control of sperm morphology has been summarized by Beatty (1970). Strain differences in sperm morphology have been studied in laboratory mice (Braden, 1959; Beatty & Sharma, 1960; Woolley & Beatty, 1967; Illisson, 1969; Woolley, 1971), rabbits (Beatty & Napier, 1960; Napier, 1961), bulls (Mukherjee & Singh, 1965) and rams (Parker, Bell & Pinjani, 1964), and the heritability of certain characteristics of spermatozoa is known to be high. The genetic base of easily recognized species-specific differences in sperm morphology is difficult to study, however, because appropriate hybrid crosses usually cannot be made or do not yield fertile F_1 offspring. The genetic control of distinctive differences in sperm morphology through the use of interspecific hybridization, can be effectively investigated by using a group of species or semispecies (Mayr, 1970) that (1) hybridize freely under labora-

tory conditions, (2) have measurable differences in sperm morphology, and (3) produce fully fertile hybrid males. Some Mexican and United States members of the salamander genus *Ambystoma* (Family Ambystomatidae) form such a group. Within this genus, there is a broad spectrum of hybridization potential and many of the hybrids are fertile (Brandon, 1972 and unpublished data; Nelson & Humphrey, 1972). We have discovered specific differences in the spermatozoa of many of these species.

In this paper, the measurements of the spermatozoa of four closely related species or semispecies of *Ambystoma* (three Mexican and one U.S.) and eight interspecific hybrid combinations are discussed. The four species, identified here by the letters *d*, *m*, *z*, and *t* respectively, are *Ambystoma dumerilii* (Dugès, 1870), the achoque, known only from Lake Pátzcuaro, Michoacán; *A. mexicanum* (Shaw, 1789), the axolotl, an inhabitant of remnants of the Lake of Mexico at Xochimilco, Distrito Federal; an undescribed species from Zacapu, Michoacán; and *A. tigrinum* (Green, 1825), the tiger salamander, a common species ranging over most of the United States and south to the Transverse Volcanic Belt of Mexico. The first three are closely related paedogenetic semispecies which are quite distinct from each other in morphology and pigmentation.

MATERIALS AND METHODS

Spermatozoa were obtained from a total of thirteen animals. Four animals were collected from their natural habitats and maintained in the laboratory until used; the rest were reared from embryos obtained in the laboratory, either through artificial insemination following methods outlined by Humphrey (1962) or through spontaneous courtship of a previously isolated pair of adults placed together in a large aquarium (Table 1).

Spermatozoa were either drawn from the ductus deferens into a capillary tube, spot-smearred from spermatophores, or taken from suspensions made by shredding the ductus deferens in 10% Lee's modified Ringer-Locke solution (Emmel & Cowdry, 1964). Spot-smears were made on slides lightly coated with albumin, fixed in 95% ethanol and stained the next day by the following techniques: Heidenhain's iron haematoxylin, Bromphenol blue, Kernechtrot (Roboz Surgical Inst. Co., Washington, D.C.)-Fast green FCF, Feulgen and periodic acid-Schiff (PAS). Lengths of relatively straight whole spermatozoa and their parts were then measured under a light microscope at $\times 400$ and $\times 1000$ using a calibrated ocular micrometer. The sinuous, coiled and perfectly straight spermatozoa were not measured as the first two could not be measured accurately and the last tended to be stretched. Measurements were taken of the lengths of the sperm head including the acrosome (Text-fig. 1), the sperm neck, and the whole spermatozoon. All measurements were recorded to the nearest 0.5 μm .

Multiple comparisons among means of the measurements were performed for the four parental species and their interspecific hybrids using the Student-Newman-Keuls test for unequal sample sizes (Sokal & Rohlf, 1969), which appeared most appropriate due to the large number of means (thirteen) to be compared, all with unequal sample sizes.

Table 1. Species, source and characteristics of the males of four species of *Ambystoma* and eight interspecific hybrid combinations whose spermatozoa were measured and compared

Species or hybrid combination	Code ⁶	Animal source or number	Source of spermatozoa	Snout-vent length (mm)	Age (months)	Postmetamorphic
<i>A. tigrinum</i>	<i>t</i>	Carbondale, IL	Ductus deferens ¹	85	?	Yes
F ₁ hybrid	<i>m/t</i> ⁵	H2 <i>m/t</i> -2	Ductus deferens ¹	105	24	Yes
<i>A. mexicanum</i>	<i>m</i> ₁	Xochimilco, D. F.	Spermatoaphore ³	120	?	No
F ₁ hybrid	<i>m</i> ₂	Xochimilco, D. F.	Ductus deferens ²	120	?	No
F ₁ hybrid	<i>m/z</i>	H7 <i>m/z</i> -4	Ductus deferens ¹	118	29	No
F ₁ hybrid	<i>z/m</i>	H7 <i>z/m</i> -6	Ductus deferens ¹	111	20	No
<i>A.</i> —(undescribed)	<i>z</i>	Zacapu, Michoacán	Ductus deferens ¹	135	?	No
F ₁ hybrid	<i>z/d</i>	H8 <i>z/d</i> -4	Ductus deferens ¹	94	18	No
F ₁ hybrid	<i>d/z</i> ⁵	H8 <i>d/z</i> -2	Ductus deferens ¹	120	28	No
<i>A. dumerilii</i>	<i>d</i>	B1-20	Ductus deferens ²	125	31	No
F ₁ hybrid	<i>d/m</i> ⁵	H2 <i>d/m</i> -6	Spermatoaphore ³	105	15	No
Hybrid	<i>m</i> × <i>t</i> ⁴ / <i>d</i> ⁵	H5	Spermatoaphore ³	115	18	No
Hybrid	<i>m</i> × <i>t</i> ⁴	Humphrey 3285-3	Spermatoaphore ³	140	?	No

¹ Seminal fluid collected by capillary tube through slit in wall of the ductus deferens.

² Ductus deferens removed and macerated to make suspension of seminal fluid in 10% Amphibia Ringer's.

³ Snear made of seminal fluid from freshly deposited spermatoaphore.

⁴ Animal of hybrid origin, but now mainly of *A. mexicanum* genome.

⁵ Spermatozoa of these hybrid crosses have successfully fertilized eggs; others are untested.

⁶ Female/male.

TEXT-FIG. 1. Schematic illustration of the *Ambystoma* spermatozoon, greatly foreshortened and exaggerated in thickness. Indicated are measured limits of the head with acrosome, and the neck; whole spermatozoa were measured from the tip of the acrosome to the end of the end-piece. Axial rod (AR), tail membrane (TM), flagellum (F) and end-piece (EP). (The limits of the mid-piece are not indicated since its length was not measured.)

RESULTS

The spermatozoa of the various species and hybrid combinations had similar morphology, apparently differing only in total length and the lengths of the major components. Our descriptions of sperm morphology follow the terminology of Baker (1962) and Fawcett (1970). The elongated, cylindrical head gradually tapers anteriorly to a point bearing an acrosomal cap. The acrosomal cap stained with Fast green FCF, but was PAS-negative. The rest of the head stained red with Kernechtrot or Feulgen. The neck (connecting piece) fits into a concave depression at the posterior end of the head, with the head extending as a sleeve over most of the neck. The sleeve stained red with Kernechtrot or Feulgen, whereas the neck stained moderately with Fast green FCF and strongly with Bromphenol blue. The tail is connected to the posterior end of the neck and consists of a supporting axial rod (= axial fibre) attached to a lateral flagellum by an undulating membrane. The tail is divisible into a mid-piece, a main-piece and an end-piece. The main-piece is easily discernible in *Ambystoma* because of the tail membrane (Martan & Wortham, 1972) or keel (Noble & Brady, 1933) extending along it opposite the undulating membrane. Both membranes terminate at the tapering end of the axial rod, but a short segment of flagellum extends beyond as the end-piece. The entire sperm tail stained strongly with Fast green FCF. The spermatozoa which were considered most reliable for measurement were those which stained with Heidenhain haematoxylin, because this stain yielded the clearest cytological detail (acrosomal termination, tail membrane, undulating membrane, end-piece).

Spermatozoa taken from the ductus deferens did not differ in morphology from those taken from spermatophores. A comparison of spermatozoa taken from two individuals of the same species (m) further showed that the source of the spermatozoa, i.e. ductus deferens versus spermatophore, also had no influence on sperm measurements. Thus, measurements of the individuals m_1 and m_2 were treated as a composite (m) when species were discussed. The means and standard errors of the three sperm characters measured for the thirteen animals are presented in Tables 2 to 4.

Of the four parental species, *A. tigrinum* had the longest spermatozoa, the longest head with acrosome, and the longest neck. The rank order of the means for the other three parental species shifted as follows: (1) head with acrosome— $t > m > d > z$ (see Table 1 for code); (2) neck— $t > d > z > m$, and (3) whole spermatozoon— $t > z > d > m$.

Table 2. Influence of interspecific hybridization on the length of the sperm head with acrosome of four species of *Ambystoma* and eight interspecific hybrid combinations

Code†	Length of head with acrosome (μm)	
	Sample size	Mean $\pm$ S.E.*
<i>t</i>	15	125.07 $\pm$ 1.10
<i>m/t</i>	8	118.56 $\pm$ 0.67
<i>d/m</i>	6	113.83 $\pm$ 1.08
<i>m</i> $\times$ <i>t</i>	5	112.90 $\pm$ 0.84
<i>m</i> ₂	6	110.42 $\pm$ 2.53
<i>m</i> ₁	8	110.06 $\pm$ 1.64
<i>m</i> $\times$ <i>t/d</i>	9	109.11 $\pm$ 0.75
<i>z/m</i>	11	106.64 $\pm$ 0.91
<i>d</i>	6	104.20 $\pm$ 1.00
<i>d/z</i>	18	103.11 $\pm$ 0.80
<i>m/z</i>	19	102.37 $\pm$ 0.83
<i>z/d</i>	16	100.53 $\pm$ 0.82
<i>z</i>	12	98.50 $\pm$ 0.46

* Measurements not significantly different at least at $P = 0.05$ are connected by vertical lines.

† See Table 1.

When the means for length of head with acrosome were tested by the Student—Newman—Keuls (SNK) technique, all four species were found to differ significantly; *m* versus *d* at $P = 0.05$, and all others at $P \leq 0.01$ (Table 2). Although *t* and *d* were not found to be significantly different from each other for length of sperm neck, they were both significantly different from *m* and *z* ($P \leq 0.01$), as was the comparison of *m* with *z* (Table 3). For the length of whole

Table 3. Influence of interspecific hybridization on the length of the sperm neck of four species of *Ambystoma* and eight interspecific hybrid combinations

Code†	Length of neck (μm)	
	Sample size	Mean $\pm$ S.E.*
<i>m</i> $\times$ <i>t/d</i>	11	14.36 $\pm$ 0.10
<i>t</i>	37	13.76 $\pm$ 0.07
<i>m/t</i>	14	13.57 $\pm$ 0.10
<i>d</i>	7	13.43 $\pm$ 0.17
<i>d/m</i>	10	13.20 $\pm$ 0.15
<i>z/d</i>	18	12.92 $\pm$ 0.06
<i>z</i>	25	12.76 $\pm$ 0.10
<i>z/m</i>	13	12.69 $\pm$ 0.16
<i>d/z</i>	14	12.57 $\pm$ 0.10
<i>m/z</i>	20	12.00 $\pm$ 0.09
<i>m</i> $\times$ <i>t</i>	7	11.50 $\pm$ 0.34
<i>m</i> ₂	7	11.36 $\pm$ 0.18
<i>m</i> ₁	14	11.32 $\pm$ 0.10

* Measurements not significantly different at least at $P = 0.05$ are connected by vertical lines.

† See Table 1.

Table 4. Influence of interspecific hybridization on the total sperm length of four species of *Ambystoma* and eight interspecific hybrid combinations

Code†	Length of whole sperm. (μm)	
	Sample size	Mean $\pm$ S.E.*
<i>t</i>	19	496.65 $\pm$ 2.30
<i>m/t</i>	13	468.65 $\pm$ 2.10
<i>d/z</i>	10	461.88 $\pm$ 2.54
<i>z</i>	12	457.50 $\pm$ 3.26
<i>m</i> $\times$ <i>t/d</i>	11	453.30 $\pm$ 1.19
<i>d</i>	8	450.78 $\pm$ 4.20
<i>m</i> ₂	8	444.70 $\pm$ 5.13
<i>m</i> ₁	10	444.10 $\pm$ 3.71
<i>z/d</i>	25	442.45 $\pm$ 1.80
<i>m</i> $\times$ <i>t</i>	4	441.88 $\pm$ 4.03
<i>z/m</i>	16	439.05 $\pm$ 2.42
<i>m/z</i>	6	436.25 $\pm$ 4.78

* Measurements not significantly different at least at $P = 0.05$ are connected by vertical lines.

† See Table 1.

spermatozoon (Table 4), *t* was different from each of the other three species ($P \leq 0.01$), while *z* differed from *m* ($P \leq 0.05$) but not from *d*, nor did *d* differ from *m*.

Head with acrosome

The hybrid, *m/t*, was different ($P \leq 0.01$) from both parental species, whereas the *m* $\times$ *t* hybrid, presumed to be largely *m* because of successive backcrossing with *m*, differed from *t* ($P \leq 0.01$) but not from *m* (Table 2). Hybrid *z/m* differed ($P \leq 0.01$) from *z* but not from *m*, whereas the reciprocal, *m/z*, differed (≤ 0.01) from both parental species. The *d/z* F_1 differed ($P \leq 0.01$) from *z*, but the reciprocal hybrid, *z/d*, did not, nor did *z/d* and *d/z* differ from *d*. The *d/m* F_1 exceeded *d* ($P \leq 0.01$) and resembled *m*; however, the F_1 of a cross between *m* $\times$ *t* and *d* (*m* $\times$ *t/d*) differed from *t* ($P \leq 0.01$), and from *d*, but not from *m*.

Thus, in every case except one (*d/m*), the mean measurements of the head with acrosome of the F_1 hybrids lay between those of the parental species. For the two situations where reciprocal hybrids were available (*d/z* and *z/d*; *m/z* and *z/m*), the differential influence of one parental genome was not definitive. In the crosses involving *d* and *z*, a maternal influence was apparent, although this was not statistically significant. For the crosses between *m* and *z*, the reverse was true, i.e. the influence appeared to be paternal in origin, and the differences were significant. The *m/t* hybrid fell approximately at the mid-point between the two parental species, in contrast to the *m* $\times$ *t* hybrid, which favoured the *m* side. This difference was probably the consequence of successive backcrossings of the original *m* $\times$ *t* hybrid with *m* for several generations, which may have resulted in a considerable recovery of the original genome, as the lack of a significant difference between these two means suggests.

The *m* $\times$ *t/d* hybrid, while falling mid-way between its parental species, was

significantly different only from its d parent, but not from $m \times t$ nor m , nor from d/m , which might reasonably have represented a reciprocal if the assumption of a sizeable recovery of the m genome were valid. Additional measurements are needed before definitive conclusions can be drawn as to differential parental influence.

Neck

Necks of the hybrid $m \times t/d$ spermatozoa were significantly ($P \leq 0.01$) longer than those of t and d , and far exceeded those of m , which were the shortest of all the necks examined. The d/m F_1 differed ($P \leq 0.01$) from m , but not from d , and both z/d and d/z differed from d ($P \leq 0.05$ and 0.01 , respectively), but not from z (Table 3). The m/z F_1 differed from both m and z ($P \leq 0.01$), whereas the reciprocal, z/m , differed from m ($P \leq 0.01$) but not from z . The hybrid, $m \times t$, largely m in genome, differed from t ($P \leq 0.01$), but resembled m as would be expected from successive back-crossing.

With two exceptions, $m \times t/d$ and d/z , the mean length of the sperm neck of the hybrids fell between the values for the parental species, but there was no definitive influence of parental genome (i.e. maternal versus paternal). In the reciprocal hybrids, z/m and m/z ; d/z and z/d , the major influence appeared to be from the z genome regardless of whether z represented a paternal or maternal contribution, since three of the four means compared with that of z failed to show significance. When d and m were involved together in crosses (d/m and $m \times t/d$), the influence of the d genome appeared to predominate, although significant heterosis was apparent for the $m \times t/d$ hybrid in that it exceeded all other measurements. For the only major cross involving t (m/t), the influence of the t genome was clearly evident. The close agreement between the means for $m \times t$ and m lends further evidence that a sizeable portion of the m genome was recovered through the successive generations of backcrossing.

Whole spermatozoa

The measurements of whole spermatozoa revealed little difference between species, with only one (t) being significantly different from all others (Table 4). Similarly, significant differences between hybrids and their parental species were few. The m/t F_1 whole spermatozoa were intermediate between those of the two parental species and differed significantly from each ($P \leq 0.01$). Those of the d/z F_1 did not differ from either parental species, but did differ from the reciprocal, z/d ($P \leq 0.01$), which in turn differed from z ($P \leq 0.01$) but not from d . Both z/m and m/z differed from z ($P \leq 0.01$), but not from m , even though mean lengths of both hybrids were lower than the value for m . The hybrid $m \times t$ did not differ from m , but did differ significantly from t ($P \leq 0.01$). Therefore, parental genome influence was not as detectable as in the measurements of the sperm head or the neck. Two of the hybrids (m/t and z/d) exhibited significant differences from both parents. For the former, the hybrid measurement fell between both parental species, while the latter measurement exhibited a heterotic effect, being smaller than either parental measurement. The reciprocal, d/z , also exhibited a possible heterotic effect, but in the opposite direction from that of z/d .

For the hybrid, *d/m*, only two whole spermatozoa were suitable for measurement and, thus, this group is missing from the comparisons. The mean measurement for these two spermatozoa was 442 μm , placing it below both parental measurements.

DISCUSSION

We realize the risk of generalizing from results taken from only one individual of each salamander stock. We are encouraged to do so, however, by (1) the slight variation among spermatozoa from a given male considering the magnitude of difference between stocks; (2) the observation that values from two individuals of *A. mexicanum* are essentially identical even though the spermatozoa came from the ductus deferens of one and from a spermatophore of the other; (3) strain-specific differences in sperm morphology in mammals; (4) our published (Martan, Brandon & Wortham, 1973) and unpublished data on taxonomic diversity of sperm morphology in another family of salamanders, the Plethodontidae; and (5) the fact that our values for hybrids lay between those of parental species even though samples of parental species came from individuals other than the actual parents.

The geographic ranges of *A. dumerilii*, *A. mexicanum* and "z", viewed in the light of Barbour's (1973) analysis of the geological and physiographic history of the Mexican Plateau, suggest speciation, from a common ancestor, of populations isolated in different portions of the disrupted Río Lerma drainage. Though Tihen (1958) placed *A. mexicanum* in a separate species group from *A. tigrinum* and both of these species in a separate sub-genus from *A. dumerilii*, he pointed out that *A. mexicanum* is virtually indistinguishable morphologically from members of the *A. tigrinum* species group and that *A. dumerilii* is generally similar to *A. mexicanum*, with unquestioned affinities to the *A. mexicanum*-*A. tigrinum* complex. Similarities of courtship behaviour, ease of hybridization, and features of hatchling larvae (Brandon, 1972) further suggest a close relationship among these four species. In sperm dimensions, *A. dumerilii*, *A. mexicanum* and "z" cluster in comparison with other examined species of *Ambystoma* (J. W. E. Wortham, R. A. Brandon and J. Martan, unpublished observations), with Illinois *A. tigrinum* being somewhat divergent. Whether or not Mexican populations of *A. tigrinum* resemble Illinois populations in sperm dimensions remains to be examined in detail; however, preliminary measurements of spermatozoa from Mexican *A. tigrinum* (collected near Mexico City) suggest shorter necks and shorter total sperm lengths, which more nearly resemble those of *A. mexicanum*. The divergence of measurements of Illinois *A. tigrinum* from those of the three Mexican species and from Mexican *A. tigrinum* may reflect geographic variation in these traits, or taxonomic difference between Mexican and Illinois populations of *A. tigrinum*. Data on sperm morphology may eventually contribute to clarification of the evolutionary and taxonomic relationships among Mexican populations of the *A. tigrinum* species group. Although they must be considered provisional, our data suggest that a more thorough analysis involving larger samples of males would be fruitful.

That these differences of sperm morphology may be under genetic influence is not unexpected, since intraspecific genetic control has been well documented in mice by Illisson (1969), Woolley (1971) and Beatty (1970).

Illisson (1969) was the first to demonstrate a significant maternal influence on sperm morphology, i.e. head shape. Although our data do not show a consistent effect of either the paternal or maternal contribution in those instances where reciprocal cross hybrids were available, they do reveal the possible influence of a species genome effect. In those crosses involving *A. dumerilii*, there was a consistent, though non-significant, tendency for measurements to favour the *A. dumerilii* genome. Similar trends were not so apparent with the other species or their hybrids, but it is not unlikely that additional information will reveal that genomic effects are much more pronounced than differential parental contributions.

The estimates of low gene number controlling sperm head shape presented by Illisson (1969) and the estimates of high heritability for both head shape (Illisson, 1969) and mid-piece length (Woolley & Beatty, 1967; Woolley, 1971) lend further evidence that genomic effects may be large for such interspecific crosses as are presented here for *Ambystoma*. If similar genetic control is effective for this group also, then the utilization of the sperm measurements for length of head with acrosome and neck length, as additional reliable taxonomic traits, is valid.

It seems possible that the species-specific morphological differences of spermatozoa may be correlated with differences in the thickness of the egg membranes to be penetrated during fertilization, as the data of Kawamura (1953) and Nelson & Humphrey (1972) suggest. Beatty & Sidhu (1971) demonstrated a correlation between the length of the spermatozoa and of the internal genitalia of both male and female in five species of *Drosophila*. Further clarification of the rôle of sperm dimensions and function must await more detailed studies of egg membranes, the process of penetration itself, and sperm morphology. Our data, however, strongly suggest that further studies of this nature in salamanders are warranted.

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