Did Paul Kammerer Discover Epigenetic Inheritance? A Modern Look at the Controversial Midwife Toad Experiments

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ABSTRACT The controversy surrounding the alleged Lamarckian fraud of Paul Kammerer's midwife toad experiments has intrigued generations of biologists. A re-examination of his descriptions of hybrid crosses of treated and nontreated toads reveals parent-of-origin effects like those documented in epigenetic inheritance. Modification of the extracellular matrix of the egg as described by Kammerer provides a plausible cause for altered gene methylation patterns. Traits such as altered egg and adult body size in Kammerer's ''treated'' toads are inherited epigenetically in other tetrapods. A preliminary model involving the environmental silencing of a maternally inherited allele can be attempted to explain the midwife toad experiments. Given available molecular tools and our current understanding of epigenetics, new experimentation with the midwife toad is strongly encouraged. *J. Exp. Zool. (Mol. Dev. Evol.) 312B, 2009*.

How to cite this article: Vargas AO. 2009. Did Paul Kammerer discover epigenetic inheritance? a modern look at the controversial midwife toad experiments. J. Exp. Zool. (Mol. Dev. Evol.) 312B:[page range].

Paul Kammerer, a renowned Lamarckian experimentalist in the early 20th century, committed suicide in 1926, shortly after an article published in *Nature* (Noble, '26) presented evidence suggesting he could have committed fraud in his experiments of inheritance of acquired traits in the midwife toad, *Alytes obstetricians*. These demanding experiments spanned several years and have never been properly re-attempted. The case remains unsolved: several different authors have considered that Kammerer's experiments were probably authentic (Koestler, '71; Gould, '72; Gliboff, 2005, 2006), but the shadow of doubt has made any citation of his work objectionable (Zirkle, '54). His entire scientific legacy nowadays is thus nonexistent, and Kammerer is more often cited as a historic example of Lamarckian scientific fraud (for a recent review on Kammerer, see Gilbert and Epel, 2008). Here, I point out some aspects of the description of Kammerer's midwife toad experiments in his book ''*The Inheritance of Acquired traits*'' (Kammerer, '24) that shows remarkable resemblances to currently known epigenetic mechanisms, which are very unlikely to have been a fabrication of Kammerer's imagination.

The experiment

The midwife toad is a species with highly terrestrial habits for an amphibian, copulating and fertilizing its eggs on land. Unlike closely related toads of more aquatic lifestyles, such as *Discoglossus* and *Bombina* (San Mauro et al., 2004), strings of fertilized eggs are not deposited in water, but rather the male of the midwife toad wraps them around his legs, and carries them on land stuck to the legs during their embryonic development (Fig. 1A). Thus, early embryos are first exposed to air: they are only delivered into the water later, upon emerging from

Grant sponsor: Government of Chile FONDECYT; Grant number: 11080258.
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Received 12 January 2009; Revised 23 July 2009; Accepted 25 July 2009
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.b.21319

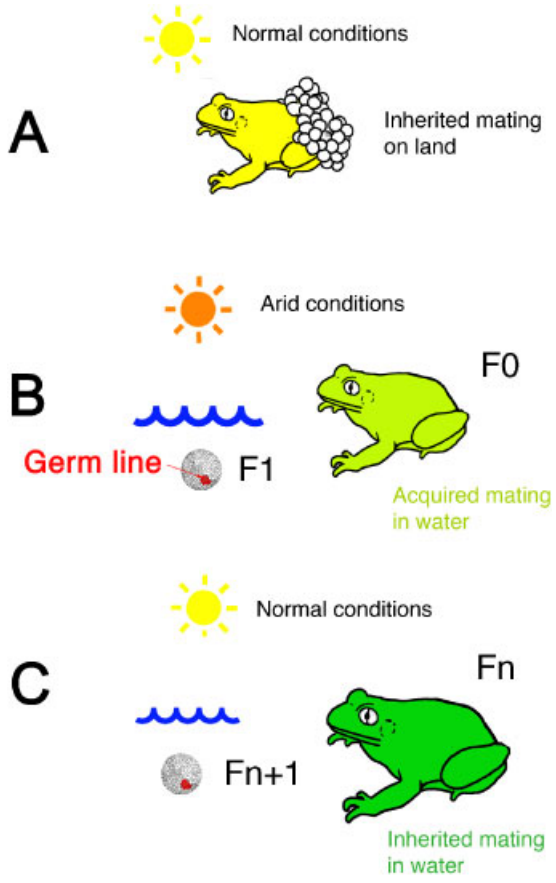

Fig. 1. Kammerer's basic experiment of ''inheritance of acquired traits.'' Normally, the midwife toad mates on land and the male carries the fertilized eggs on his legs during embryonic development, which is spent exposed to air (color coded as a yellow toad). By artificially exposing midwife toads to a heated, dehydrating environment, they could be made to spend most of their time in cool water, where they now copulated and fertilized their eggs (light green toad). The eggs would cease to be carried by the male, but remained in the water. A few of these water eggs survived and developed into an F1 of toads that innately preferred to mate and lay their eggs in water, even if normal non-arid conditions are restored (green frog). Kammerer bred up to F6 of ''water'' toads with this inherited preference. From F1 onwards, both somatic (in gray) and germ-line cells (in red) were exposed to water during embryonic development. ''Water toads'' developed a larger adult body size and from F3 onwards, males developed nuptial pads in the reproductive season.

the egg as tadpoles (Raxworthy, '90). In closely related species of aquatic toads, males develop nuptial pads on their forelimbs and fingers during the mating season. These are rough, pigmented epidermal thickenings that help grasp the slippery female during copulation in water. In the terrestrial midwife toad, nuptial pads are absent.

Beyond the more controversial aspects of past Lamarckian thinking (such as the recurrent connection to vitalism), the phrase ''inheritance of acquired traits'' can be used to describe the empirical situation in which a modified phenotype is environmentally induced and then observed to persist in the progeny, despite the progeny's not having been exposed to the same environment. Paul Kammerer reported having artificially exposed midwife toads to an experimentally overheated, arid environment, while at the same time providing them with a basin of cool water. Under these conditions, the toads now spent most of their time in the water, where they would now copulate and fertilize their eggs. These eggs were deposited directly in the water, and were no longer carried by the male (Fig. 1B). Only a few (3–5%) of these ''water'' eggs lived on to hatch into tadpoles, and developed into an adult toad that, along with certain morphological differences, preferred to copulate in water even under normal (nonoverheated) conditions, no longer taking care of the eggs (Fig. 1C). This was the basic argument for the inheritance of an acquired behavioral trait. Kammerer continued to breed these ''water toads'' up to generation F6 throughout which copulation in water persisted. By generation F4, Kammerer reported that ''water'' males were developing noticeable nuptial pads during the mating season (upon re-examination, Kammerer found that the rudimentary nuptial pads were already present at F3). Across the successive generations of water toads, Kammerer described the gradual intensification of other traits as well: the amount of yolk in eggs decreased and the thickness of the gelatinous cover increased; the gills in tadpoles extended from the first branchial arch only, to all the three arches in later generations. Additionally, Kammerer performed hybrid crosses of treated and untreated toads, from which he reported obtaining roughly Mendelian proportions of ''water'' to ''land'' toads (Kammerer, '11; as cited in Gliboff, 2006; Kammerer, '24). He thus argued that his experiments involved inheritance by means of ''true'' Mendelian genes.

The fraud controversy

Some contemporaries of Kammerer, notably the early geneticist William Bateson, implicitly questioned Kammerer's honesty by expressing their disbelief that Kammerer had ever obtained nuptial pads in *Alytes* (Bateson, '19). In response, Kammerer ''toured,'' displaying specimens of his ''water toads'' to any of his colleagues willing to examine them (Koestler, '71). After the ravages of

World War I, only a single preserved male specimen with nuptial pads remained. The herpetologist G. Kingsley Noble examined the nuptial pads of this specimen, discovering by dissection and chemical analysis that the toad forelimbs had been injected with India ink. Noble thus concluded that the existence of nuptial pads was ''a matter of conjecture'' (Noble, '26). Kammerer denied the implicit accusation of fraud, claiming somebody else must have injected the specimen with ink. Unfortunately, no one has ever seriously reattempted to breed the midwife toad in captivity; the experiment thus has never been repeated that would put to test the veracity of Kammerer's results. In 1971, Arthur Koestler published his famous book, ''*The Case of the Midwife Toad*.'' In this book Koestler argued that fraud was unlikely because too many reputed scientists had observed the nuptial pads and even the experiments themselves in progress (Koestler, '71). Koestler also pointed out an important biological fact: a specimen of midwife toad had been collected in the wild that presented nuptial pads (Kandler, '24). Thus, midwife toads do have the potential for developing nuptial pads.

Rather than focusing exclusively on the nuptial pads and the controversy surrounding them, it can be more helpful to pay attention to other changes reported by Kammerer, in order to examine in modern terms whether a mechanism exists that could potentially underlie these observations. In recent years, evidence has accumulated on how genes can be modified by the extra-organismal environment through mechanisms such as DNA methylation. Transgenerational persistence of gene methylation in the germ-line (Guerrero-Bosagna et al., 2005; Gilbert and Epel, 2008) can further explain observed instances of ''inheritance of acquired traits'' (John and Surani, '99; Jablonka and Lamb, 2005; Akimoto et al., 2007; Jirtle and Skinner, 2007). Taking all these facts into consideration, I reexamined Kammerer's last book (*The Inheritance of Acquired Traits*, 1924), in which he summarizes his experiments with the midwife toad. Here, I point out some striking similarities to currently known epigenetic mechanisms that have not been previously discussed regarding the Kammerer controversy.

RESEMBLANCES TO EPIGENETIC MECHANISMS

Parent-of-origin effects

As mentioned earlier, Paul Kammerer had performed hybrid crosses between treated and nontreated toads, reporting a Mendelian phenomenon of dominance (Kammerer, '11, '24; cited in Koestler, '71). In the F1, the unchanged, ''land'' phenotype was dominant, present in all the toads. Accordingly, when crossing two F1 toads, the resulting F2 generation presented a rough 3:1 ratio dominance of water to land phenotypes. However, an interesting detail is mentioned by Kammerer ('24, p 99):

> To be sure, a certain complication arises, inasmuch as the dominant characteristic (i.e.) the one preponderant in all children, and 3/4 of grandchildren, *follows the father and, for this reason, a change of dominance from the normal to the changed form is to be observed, depending on whether a normal specimen or a changed specimen plays the part of the father*.

This ''complication'' (illustrated in Fig. 2) must have been quite startling to Kammerer and his contemporaries, but not nowadays, given our current knowledge of non-Mendelian genetics. Kammerer immediately continues to describe the difference with Mendel's discoveries, that is the namesake of non-Mendelian genetics: ''In other cases, it is of no importance which race in crossing experiments is employed on the part of the father, and to which the part of the mother is apportioned.''

This effect of parental sex on the dominance of traits is known as a ''parent-of-origin effect,'' and is well known in the case of imprinted genes. Depending on whether a certain allele is exposed to the cellular environment of either the male germ-line, or the female germ-line, it becomes epigenetically silenced or not, for instance, by CpG methylation. (Razin and Cedar, '94; Bartolomei and Tilghman, '97; Constancia et al., '98; Costello and Plass, 2001; Surani, 2001; Wood and Oakey, 2006; Ideraabdullah et al., 2008). Shortly after fertilization, a generalized de-methylation phase erases methylations on most genes. Because the development of different cell types involves different methylation patterns (Rottach et al., 2009), this ''reset'' presumably allows totipotentiality of the cells of the early embryo. However, in the case of imprinted genes, methylations acquired in the germ-line persist through the de-methylation phase, affecting the phenotype of the embryo. For a given allele that is silenced by methylation only in the female germ-line, the phenotype of the

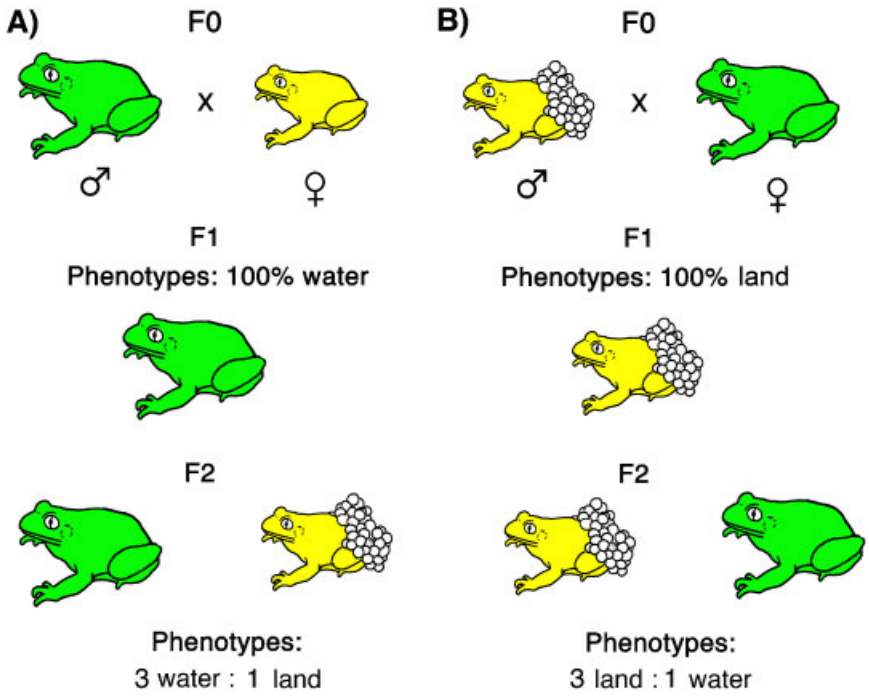

Fig. 2. Parent-of-origin effects in Kammerer's hybrid crosses. Kammerer reported that hybrid crosses of ''water'' and ''land'' toads rendered a typically Mendelian phenomenon of dominance, with 100% dominant phenotypes at F1 and a rough 3:1 ratio of dominant to recessive phenotypes at F2. However, Kammerer also reported a complication: either the land phenotype, or the water phenotype, would be dominant depending on which phenotype was present in the male used in the hybrid cross, such that if the male is a water toad, the water phenotype dominates (**A**), whereas if the male is a land toad, the land phenotype dominates (**B**). This parent-of-origin effect is currently well known in non-Mendelian genetics, in which the passage of an allele through the germ-line of one of the sexes only (paternal or maternal) determines the inactivation of the allele in the progeny. This occurs in genomic imprinting by DNA methylation as well as in experiments of environmental modification of epigenetic inheritance. Kammerer had no good reason to make up this complication, which did not contribute to his Lamarckian ideas.

progeny will depend on the allele inherited from the father. The reverse is true if an allele is only silenced in the male germ-line (the mother's phenotype is now dominant). Methylations on imprinted genes eventually do become erased in a second de-methylation phase that occurs in the early development of germ-line precursor cells; this allows imprinted genes to become differentially methylated during gonad sex determination, according to a male or female germ-line identity[1] (Reik et al., 2001; Edwards and Myers, 2007).

It is worth emphasizing that the reported parent-of-origin effects only complicated the scenario for Kammerer, increasing suspicions about his data (no such thing as modification of Mendelian alleles was accepted at the time), while at the same time failing to contribute in any way to his Lamarckian views.

The combination of environmentally modified inheritance and parent-of-origin effects

Parent-of-origin effects have recently been observed in epigenetics experiments involving phenotypes that are modified by the extra-embryonic environment, a combination also found in Kammerer's experiments. Exposure to either the cell environment of the male or female germ-line, combined with exposure to an environmental factor, may determine whether a gene becomes methylated or not, and/or whether this methylation will persist through the de-methylation phases, affecting the phenotype of nonexposed progeny. Exposure of a pregnant female to Vinclozolin (an endocrine disruptor) alters the phenotype of its progeny; the altered phenotype (spermatogenic defects, male infertility, breast

[1]Many imprinted genes are known in which a great amount of differential methylation of paternal vs. maternal alleles occurs after fertilization (in the embryo) rather than the germ line. In such genes, only a few ''key'' sites are differentially methylated during the development of germ line cells that do not become demethylated in the early embryo. These are thought to preserve the parental identity of alleles and determine differential methylation thereafter in the embryo (Brandeis et al., '93; Constancia et al., '98).

cancer, kidney disease, prostate disease, and immune abnormalities) is present in both males and females grown exposed to Vinclozolin, but only the males inherit the phenotype into non-exposed generations. Accordingly, altered methylation patterns are only stabilized in the germ-line of the male (Anway et al., 2005). Another recent experiment (Cropley et al., 2006) has presented evidence that an allele's parent of origin can determine whether exposure of the embryo to a special environment will have a phenotypic effect or not: the Agouti viable yellow (A^{vy}) allele is differentially methylated in mice, affecting coat color (Wolff et al., '98; Morgan et al., '99). A special diet of methyl donors in a pregnant F0 mother will show a shift in exposed F1 progeny to more methylated, darker-coated A^{vy} phenotypes, only if the F1 progeny have inherited the allele from the father. This suggests that previous passage of the allele through the paternal germ-line allows its methylation upon subsequent exposure of the embryo to methyl donors. The sex of the germ-line also affects the persistence of acquired methylations on the A^{vy} allele through the demethylation phases: only an F1 mother will inherit the acquired shift to darker coats into its non-exposed F2 progeny (Cropley et al., 2006).

These recent experiments show a combination of "parent-of-origin" effects with both environmentally modified phenotypes and their inheritance. The presence of this combination in Kammerer's experiments as well suggests that these could result from similar underlying epigenetic mechanisms.

The extracellular matrix of the egg and gene methylation

A specialized extracellular matrix surrounds the eggs of vertebrates. This matrix is sometimes named the chorion in fish, vitelline envelope in amphibians, perivitelline envelope in reptiles and birds, and the zona pellucida in mammals. This cover presents a conserved ultrastructure of fibrous matrices and glycoproteins ("ZP glycoproteins"), as well as conserved functions, such as binding to sperm during fertilization, and protection of early development (Goudet et al., 2008). The gelatinous cover of the egg in aquatic species of toads quickly swells upon being laid in water. According to Kammerer this does not occur in normal reproduction of the midwife toad. Rather, in contact with the air, the cover of midwife toad eggs becomes sticky, presumably helping the male to carry them on his legs. In Kammerer's experimental toads, the cover of the eggs became swollen and gelatinous upon contact with water. As mentioned before, only a few of these eggs would survive (around 3–5%; Kammerer '06, cited in Koestler '71). Recent experiments in the mouse have shown that removal of the zona pellucida (mechanically or by exposure to pronase) immediately after fertilization causes a reduction in DNA methylation at the two- and four-cell stages (Ribas et al., 2006) as well as affecting embryo survival (Ribas et al., 2005). Thus, the altered extracellular matrix described in Kammerer's experiment provides a possible cause for abnormal gene methylation in the water eggs.

Suggestive phenotypes

In mammals, gene methylation has been shown to be involved in the determination of adult body size; genomic imprinting is the reason why numerous hybrids between mammalian species, including goats, cats, camels, foxes, and horses lead to important differences in the adult body size of hybrid offspring, depending on whether a male or female of one or the other species is utilized (Gray, '72; Vrana et al., '98). Many imprinted genes contribute to growth, either as growth factors or growth inhibitors (Butler, 2002). Parent-of-origin effects have also been observed for traits such as the size of the egg in birds, suggesting genomic imprinting affects this trait (Tuiskula-Haavisto and Vilkki, 2007). Kammerer reported that the experimental "water" toads developed a notably larger adult body size than untreated land toads. He also reported that eggs of frogs grown in water were smaller, containing less egg-yolk content and emerging at an earlier stage from the cover of the egg. Given that imprinted genes in other tetrapods affect these traits, these changes as reported by Kammerer in the midwife toad also suggest that epigenetic inheritance could be involved.

A PRELIMINARY MODEL TO EXPLAIN THE MIDWIFE TOAD EXPERIMENTS

From the observations above alone, it becomes apparent that rather than being a fraud, Paul Kammerer could be the true discoverer of non-Mendelian, epigenetic inheritance. Here, I present a preliminary model based on current knowledge of epigenetics as an attempt to explain the midwife toad experiments as described by Kammerer in his 1924 book. The model is necessarily speculative, considering there is no new experimental data on the midwife toad, and that our general under-

standing of epigenetic mechanisms (including DNA methylation) is still underway. Further, it is yet to be contrasted with the more detailed descriptions in Kammerer's original papers on the midwife toad. Thus, it must be clearly understood that the case for Kammerer's innocence does not hinge on the details and assumptions of this preliminary model. The main purpose of the preliminary model is to illustrate how, in the context of our modern knowledge, a working hypothesis for a mechanistic explanation can be attempted for phenomena that were utterly mysterious to Kammerer and his contemporaries.

Hybrid crosses

The preliminary model presented here is based on the possibilities brought up by recent experiments with the A^{vy} allele in mice (Cropley et al., 2006). As mentioned above, environmentally induced methylation of the A^{vy} allele occurred only if A^{vy} was inherited from the F0 father. When the A^{vy} allele was inherited from the mother, the special methyl-donor-rich diet of the F0 pregnant mother had no effect on the phenotypes of the exposed F1 progeny. Thus, environmentally induced methylation of the A^{vy} allele requires previous exposure of that allele to the cell environment of the male germ-line. A similar phenomenon may have occurred in Kammerer's experiment. Let us consider that in Kammerer's experiment, a dominant allele ''A'' determines a land phenotype. ''A'' becomes environmentally silenced in early embryos exposed to water, only if these embryos have inherited the ''A'' allele from the mother. In other words, ''A'' can be silenced by exposure to water only if it has previously passed through the female germ-line (Fig. 3A). Let us also assume that Kammerer collected individuals carrying a rare, recessive ''a'' allele (this could explain the specimen with nuptial pads found in natural conditions as a rare occurrence of a double recessive). The ''a'' allele would be functionally equivalent to a silenced, nonfunctional A^x allele, with no functional alleles determining a ''water'' phenotype.

In his hybridization experiments, Kammerer could have taken for his F0, an aa water male, and an AA land female. As the male is a water toad, we can assume that the eggs were left in the water (however this cannot be confirmed from the description in Kammerer's book). All embryos of this F1 generation would thus be Aa heterozygotes. During embryonic development, both somatic and germ-line cells of the F1 embryos will grow exposed to water (rather than air, as in normal land toads).[2] In all embryos, the A allele is inherited from the mother and thus becomes silenced upon exposure to water. As a result, all F1 embryos present an $A^x a$ epigenotype and develop into water phenotypes (Fig. 3A). Consider now the cross of F1 water toads; taking into account that most methylations are erased shortly after fertilization, the resulting F2 genotypes will be 1/4 AA, 1/4 Aa with a paternally inherited A allele, 1/4 Aa with a maternally inherited A allele, and 1/4 aa. As both F1 parents are water toads, the F2 eggs would grow exposed to water, thus producing 1/4 A^xA, 1/4 Aa, 1/4 A^xa, and 1/4 aa epigenotypes, leading to 1/2 land phenotypes and 1/2 water phenotypes. This could be close enough to the rough 3:1 proportion reported by Kammerer for the F2, considering he was working in a nonmodel species with necessarily low numbers (Fig. 3A). However, the proportion would be shifted to a greater amount of water toads in the F2 (closer to the 3:1 reported by Kammerer) if some of the alleles silenced in the male germ-line of F1 toads would remain silenced beyond the ''formatting'' phase after fertilization that usually erases epigenetic markings, thus persisting into the F2 generation (Fig. 3B).

The proposed model is also consistent with the described crossing of a land AA male with a water aa female (Fig. 4). The resulting F1 generation would be Aa, with the dominant allele having been inherited from the father, and thus would develop a land phenotype. Both male and female F1 toads would have land phenotypes and mate on land. Therefore, F2 eggs would not be exposed to water. The resulting genotypes would be 1/4 AA, 1/2 Aa, and 1/4 aa, a typically Mendelian 3:1 proportion of land to water toads, as reported by Kammerer (Fig. 4).

The controlling experiment

In the basic experiment shown in Figure 1, Kammerer did not remove exposure to water from F2 and further generations (probably, eggs with a gelatinous water-swollen cover cannot survive being taken out of water and then raised in

[2] In Kammerer's experimental toads, all development from fertilization to eclosion of the tadpole stages was spent exposed to water instead of air. This extensive time frame is likely to have included the period relevant for altering methylation in the germ line. In mouse, the period in which germ cells reset their epigenetic marks and differentiate encompasses stages E8.5 (when the neural tube is not yet closed) to E15.5, during which exposure to methyl donors can alter germ-line methylation (Cropley et al., 2006).

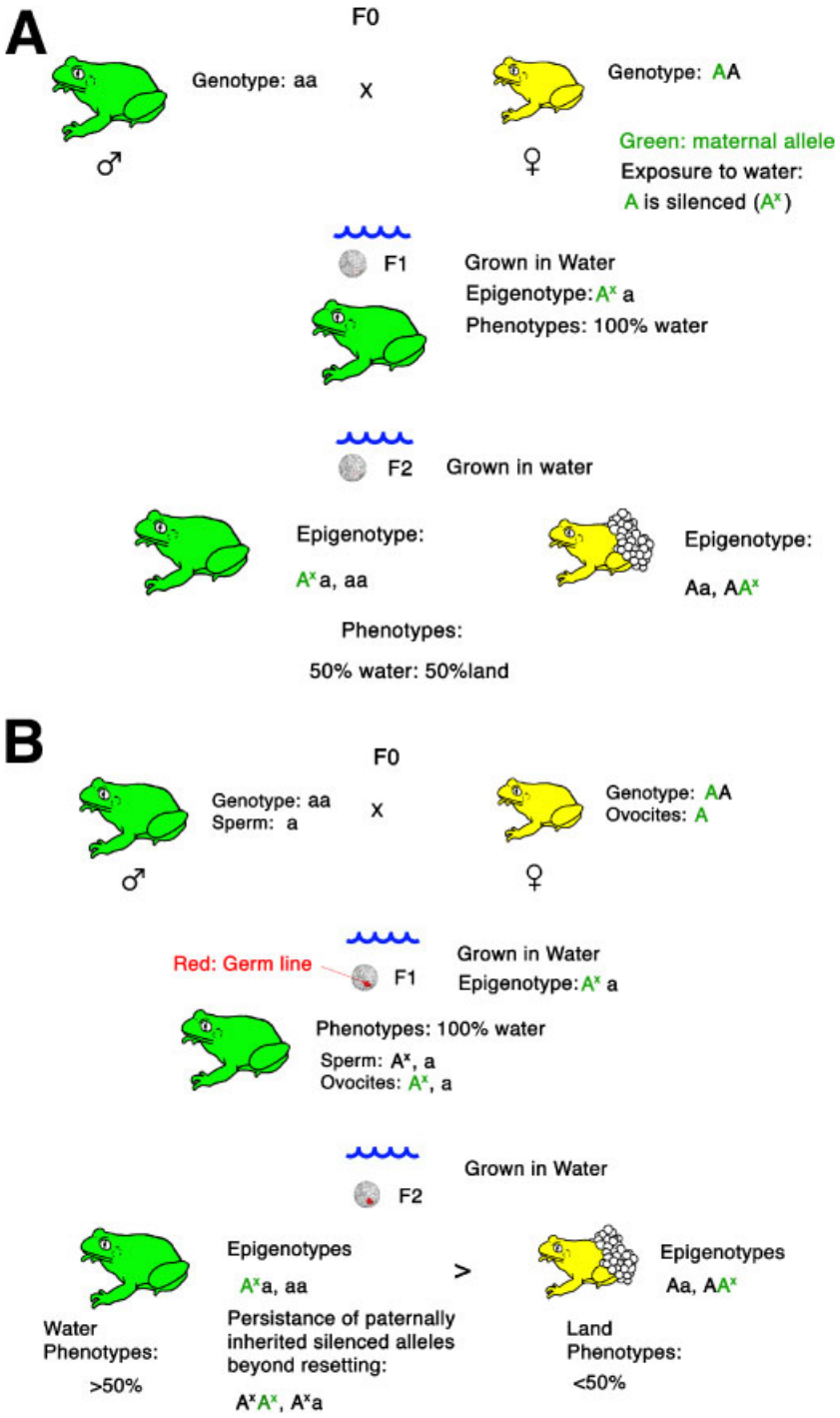

Fig. 3. A preliminary epigenetic model for Paul Kammerer's hybrid cross using a male "water" toad. (**A**) The homozygote for the recessive allele "aa" determines a water phenotype; the dominant "A" allele determines a land phenotype. "A" is epigenetically silenced (A^x, functionally equivalent to "a") only if it is inherited from the mother and then exposed to water, as in F1. Grown in water, and presenting only maternally inherited "A" alleles, all F1 will develop with an $A^x a$ epigenotype and and thus will develop a "water" phenotype. Considering the "resetting" phase shortly after fertilization that erases most epigenetic markings in the embryo, the cross of two F1 water toads should result in an F2 with 50% of water phenotypes and 50% of land phenotypes. (**B**) The same model as in (A), considering that the silenced epigenotype of some A alleles can persist beyond the resetting phase. In F1 embryos, both somatic and germ-line cells silence their "A" alleles: if some of the silenced A^x alleles in the sperm of the F1 can persist silenced beyond the resetting phase, a shift to more F2 water phenotypes would occur, greater than the expected 50% if epigenotypes were completely reset, as in Figure 1A. Kammerer reported a rough 3:1 dominance of the water phenotype in the F2.

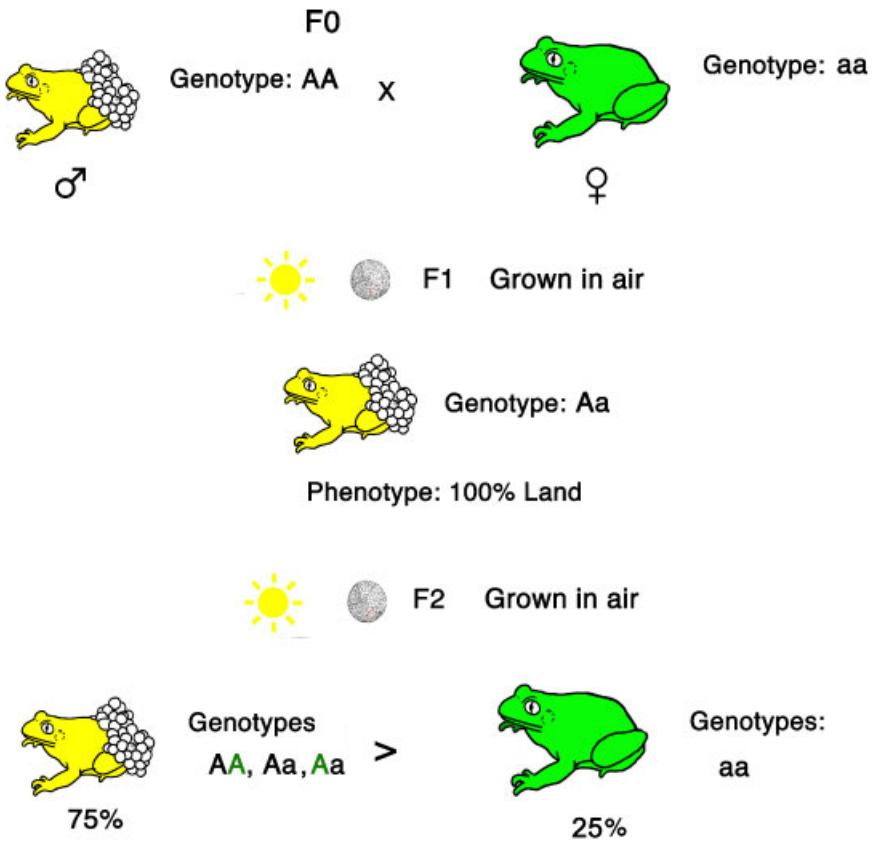

Fig. 4. The preliminary model presented in Figure 3 is also consistent with the hybrid cross in which the male is a land toad. The male land toad carries the eggs and is of AA genotype. The mother ''water toad'' is aa. All ''A'' alleles of the F1 are inherited from the father and are not silenced. The F1 toads have an Aa genotype and develop land phenotypes. The F2 are grown exposed to air: no ''A'' are not silenced and thus F2 presents a typically Mendelian 3:1 result for a heterozygote cross.

air). Because water was not taken away at F2, Kammerer's critics argued that the phenotype was not inherited, but could be a purely somatic response to repeated water exposure. If so, the development of a land or water phenotype would involve nothing but phenotypic plasticity upon exposure to either air or water, respectively. Kammerer responded by pointing to the results of his hybridization experiments, which proved that Mendelian germ-line factors were involved. Additionally, he presented a decisive ''control'' experiment: he took the recently fertilized eggs from the legs of a land toad and grew them in water. The resulting toads would prefer to mate on land and carry their eggs, despite having spent their development exposed to water (Fig. 5; Kammerer, '24).

This is yet another experiment of Kammerer's that was greatly puzzling to his contemporaries. However, the preliminary epigenetic model proposed above provides a modern explanation for Kammerer's ''controlling'' experiment. Considering that both the male and female F0 of the controlling experiment had AA genotypes, upon being grown in water, only the allele inherited from the mother would become silenced, resulting in A^xA epigenotypes and, thus, an F1 of 100% land phenotypes (Fig. 5). The main difference between this experiment and Kammerer's basic experiment of inheritance of acquired traits is that in the latter, fertilization took place in water, suggesting this is a crucial difference. The relevance of underwater fertilization is not unreasonable, given how the extracellular matrix of the egg is involved in fertilization and protection of the early embryo. As an accessory hypothesis of the preliminary model, we may consider that when fertilization takes place in water, only sperm that carries an inactive ''A^x'' or ''a'' allele can lead to a viable embryo (Fig. 6). This is consistent with the hypothesis of involuntary selection that has been argued by several authors in relation to low F1 egg survival in Kammerer's experiments (Gould, '72; Gliboff, 2006). Combined with the assumption

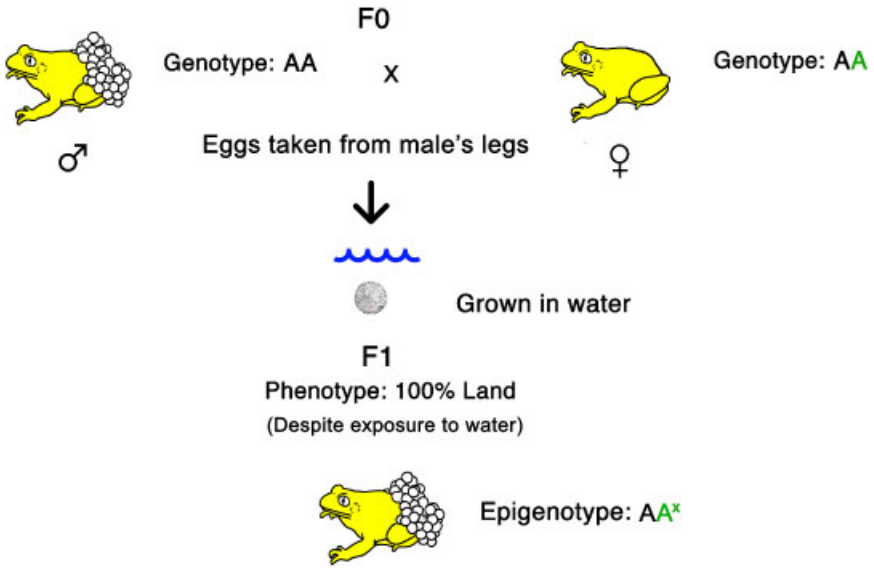

Fig. 5. Kammerer's controlling experiment. Kammerer took recently fertilized eggs from the legs of land toads and grew them in water, obtaining land toads. According to the model presented in Figure 4, only the maternally inherited allele becomes silenced upon exposure to water. The presence of the non-silenced, functional paternal allele can thus explain the development of a land phenotype despite exposure to water. This experiment reveals a crucial difference with the case in which fertilization takes place in water, which results in "water" phenotypes.

that Kammerer had isolated a rare recessive allele in his initial population, when fertilization takes place in water, only the few land males carrying the rare "a" allele would have produced viable eggs (Fig. 6). The few surviving F1 eggs would carry a paternal, inactive "a" allele: the maternally inherited allele could be a dominant "A," but would become inactivated upon exposure to water. As reported by Kammerer, the few toads Kammerer obtained in the F1 generation would all develop a "water" phenotype (Fig. 6). Consistent with this accessory hypothesis, Kammerer reported that the survival of F2 water eggs was much higher (as cited by Koestler, '71), which would greatly increase the frequency of "a" alleles. This would also explain how double homozygote "aa" water toads were frequent and readily available for Kammerer's hybridization experiments.

Trans-generational intensification of traits

As mentioned in the introduction, Kammerer described the gradual intensification of "water" traits across successive generations of water exposure. The situation could be similar to that of the A^{vy} allele of the mouse, where greater methylation (on more CpG sites) leads to darker fur-coats. In the context of the preliminary model above, the intensification of the water traits suggests an accumulation of acquired methylations that persist through both de-methylation phases. If all acquired methylations were erased in the germ-line, accumulation should not be possible.

CONCLUSIONS/PERSPECTIVES

The consistency with currently known epigenetic mechanisms, especially regarding the parent-of-origin effects, provides new compelling biological arguments in favor of the authenticity of the midwife toad experiments. Rather than committing fraud, it seems that Kammerer had the misfortune of stumbling upon non-Mendelian inheritance at a time in which Mendelian genetics itself was just becoming well accepted. The alleged "criminal evidence" for fraud in the midwife toad experiments is far from conclusive, as it does not constitute proof that the nuptial pads were never present in the experimental toads. Kammerer published photographs, including histological sections, of the nuptial pads; despite accusations, these have never been shown to correspond to *Bombina* or any other amphibian normally showing nuptial pads (Koestler, '71). Hans Przibram, a close colleague of Kammerer, maintained that

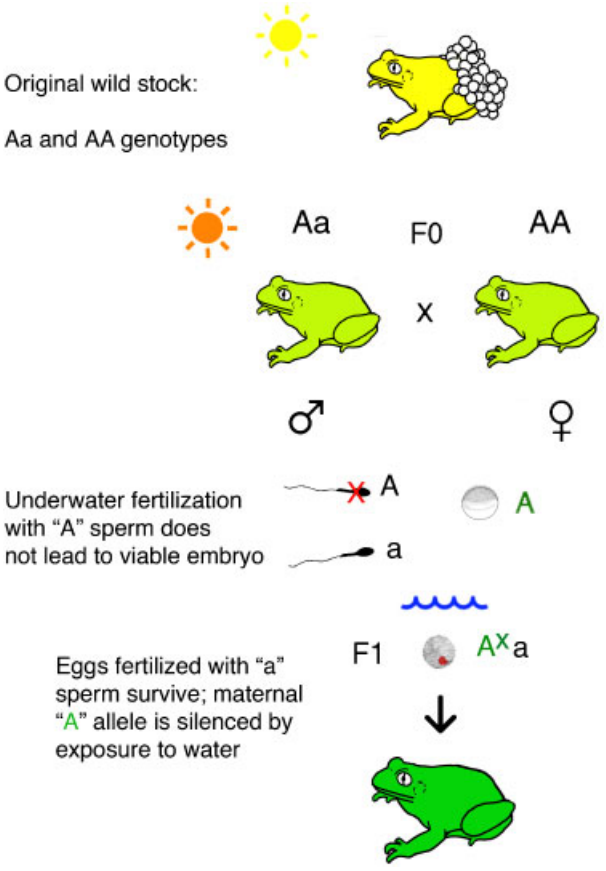

Fig. 6. A hypothesis on the difference of fertilization as exposed to water or air in Kammerer's basic experiment (Fig. 1). When exposed to water, only eggs fertilized with sperm carrying an inactive ''a'' allele can develop to term. Only a few individuals in Kammerer's initial population of land toads would carry the rare ''a'' allele. This may explain the low rate of egg survival described by Kammerer in F1. The few surviving F1 toads would carry an ''a'' paternally inherited allele and a maternally inherited ''A'' allele that is silenced upon exposure to water, developing the ''water'' phenotype.

stratification and relative nuclear sizes in these sections specifically match those of normal *Alytes*[3] (Przibram, '27). As pointed out by Kammerer and his contemporaries, nuptial pads in the midwife toad are an atavism. Based on the discovery of the naturally occurring specimen with nuptial pads, Stephen Jay Gould argued that the genes necessary for their development must still be present in the midwife toad (Gould, '72).

The biology of amphibians is also consistent with Kammerer's experiments; amphibians are known to show remarkable phenotypic plasticity, such as environmental determination of sex and facultative metamorphosis in some species (Wells, 2007). In the classic cloning experiments of King and Briggs, the phenotypes of the cloned frogs reflected the cellular origin of the nuclei, showing that imprinted memory exists in the anuran genome (King and Briggs, '56). In froglet limb regeneration, Shh expression correlates with methylation of the Sonic-Hedgehog enhancer (Yakushiji et al., 2007), suggesting that methylation is relevant for anuran development. Most important, parent-of-origin effects on body and head size occur in crosses between the bullfrog and the minkfrog (Elinson, '77), consistent with Kammerer's observations on body size in his hybrid crosses.[4] In all, the data above suggest a great potential for the study of epigenetic inheritance in amphibians, involving methylation and/or other epigenetic mechanisms.

Despite the many compelling historical and biological observations in favor of Kammerer's innocence, no greater amount of argumentation will be as decisive as new experimentation with the midwife toad, which can now be carried out with the benefit of modern molecular knowledge and tools. For instance, embryos grown in water can be checked for epigenetic processes (such as heterochromatin formation, DNA methylation, histone modifications, RNAi-dependent gene silencing, etc.) that should differ from those of normal embryos grown exposed to air. If Kammerer's data are indeed correct, the midwife toad holds the potential of becoming a crucial model system for advancing our knowledge of epigenetics and, especially so, of its evolutionary implications.

ACKNOWLEDGMENTS

Thanks to Günter P. Wagner for encouraging my pursuit of this topic. Special thanks to Jorge Mpodozis and Carlos Medina, for teaching me about ''Lamarckian'' experiments and keeping my mind open.

[3]Przibram ('27) also maintained that the histological features were identical to those of the midwife toad specimen with rudimentary nuptial pads found in nature by Kandler ('24); however, he did not publish photographs of Kandler's specimen.

[4]Parent-of-origin effects are known in nematodes, insects, fishes, amphibians, and birds (Morison and Reeve, '98). Genomic imprinting is well known in insects, mammals, and angiosperms, where common mechanisms involved (such as transcriptional silencing and DNA methylation) suggest a single ancient origin of imprinting (Anaka et al., 2009). In birds, quantitative trait loci (QTL's) for parent-of-origin effects map to clusters of genes that are orthologous to imprinted clusters in mammals (Tuiskula-Haavisto and Vilkki, 2007). These clusters also show asynchronous DNA replication, a hallmark of imprinted chromosome regions (Dünzinger et al., 2005).

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