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AMBISEXUALITY AND SEX DIFFERENTIATION IN
AMBYSTOMA¹

WINIFRED M. GILBERT

Recent and extensive use of *Ambystoma* for experimental work in sex differentiation necessitates knowledge of the normal development of their gonads. From the work of Burns, Humphrey, and Witschi, it has become quite clear that often in male salamanders a considerable cortical development accompanies that of the medullary testicular differentiation. Such ambisexual and hermaphrodite features may be expected to have either genetical or environmental causes. Genetical differences can be studied by raising groups of individuals from different localities under identical laboratory conditions. On the other hand, physiological effects of the environment should be brought out if genetically identical lots are raised under different laboratory conditions.

One factor not hitherto controlled in much of the experimental work with *Ambystoma* and which is known to be of importance in Anuran development (Witschi 10, 11, 14, Piquet 8), is temperature. In this study we have traced the developing gonads of several local races of *Ambystoma* at both room temperature (22° C., rising to about 28° toward metamorphosis) and at a temperature considerably lower, 13 ± 2° C., in order to test the influences which this factor exerts on development and sex differentiation.

Quite naturally, attention is given here especially to those species and races which have served the above-mentioned authors in their experimental studies in problems of sex differentiation and sex reversal.

MATERIAL AND METHODS

For this study eggs and embryos of *Ambystoma tigrinum* and *Ambystoma maculatum* were obtained from several widely separate localities: (1) *Ambystoma tigrinum*, from the vicinities of Iowa City, Iowa; Chicago; and Minnesota; (2) *Ambystoma maculatum* from New Haven, Connecticut; New Orleans, Louisiana; Northampton, Massachusetts, and Imboden, Arkansas.

¹ Aided by grants from National Research Council, Committee for Research in problems of sex; grant administered by Professor Emil Witschi. The writer wishes to express her appreciation to Dr. Witschi for suggesting the problem and for his helpful advice and criticism during the progress of the investigation.

When eggs arrived at the laboratory, they were removed from the jelly and one-half of them placed in tanks at a temperature of $13 \pm 2^\circ$ C., while the other half was left at room temperature. The larvae and metamorphosed salamanders were fed daily on liver.

From time to time individuals were measured and preserved. Camera lucida drawings of all gonads were made at the time they were removed from the body cavity. Zenker's solution with acetic acid was used as a fixative. Individuals were measured before fixation in Zenker's and again after being fixed and placed in 80 per cent alcohol. At room temperature *Ambystoma maculatum* shows an average shrinkage of 5.1 mm. (9 per cent); the same species in cold temperature shows an average shrinkage of 5.5 mm. (8 per cent). The room temperature *Ambystoma tigrinum* show a shrinkage of 12 mm. (9 per cent) while those in cold temperature show an average shrinkage of 9.3 mm. (8 per cent). These data refer to specimens at the metamorphosis stage. Sections were cut 10μ in thickness and stained with Delafield's hematoxylin and Congo red.

OBSERVATIONS

- I. *Ambystoma tigrinum* (Green), a differentiated race² from Iowa, Minnesota and Illinois.

a. Room temperature series

In a group of Iowa tiger salamanders, the first individual (110 mm.) metamorphosed at the age of three months. All of this group had metamorphosed by September when four and one-half months old. The average length of these animals was 145 mm. After sectioning they were examined to determine the sex ratio and the degree of ambisexuality in both larval and completely metamorphosed males.

This group consisted of 77 females + 63 males, a result which is close to the expected 1:1 ratio for a differentiated race.

In three weeks old larvae of from 20-25 mm. the sexes could not be distinguished macroscopically and when the gonads were preserved and sectioned they were found to be indifferent.

The indifferent gonad of urodeles varies somewhat from that described by Witschi (10, 15) for Anurans. In the latter the germ cells are arranged in a single layer along the peritoneum, thus forming a peripheral germinal epithelium, composed of germ cells with

² The terms "differentiated race" and "semi-differentiated race" are used here as explained by Witschi (15). The first type is purely gonochoristic, or nearly so; the second type shows regularly hermaphrodite features.

their follicles and stroma cells. On the other hand, in all types of *Ambystoma* germ cells are more scattered throughout the whole indifferent gonad.

In larvae of 30-35 mm. and 4-5 weeks of age, sex could not safely be determined without sectioning. At this time differentiation was taking place. Figure 1 shows a 32 mm. male, four weeks of age. About half of the germ cells are included in the medullary cords, but many still lie in the cortex, around the crest of the gonad. In some cases a number of germ cells within the cortex show spireme-like arrangement of the chromatin (Leptotene) and can therefore be considered as ovocytes. The medulla at this stage is a compact mass of tissue containing germ cells with their follicles. The primordium of the fat body is small and consists chiefly of blastemic cells, and quite commonly includes stray germ cells. No fat vacuoles appear at this stage. Females of this age and length are well differentiated and the germ cells show first leptotene stages.

In metamorphosing males of 10-12 weeks, averaging 100 mm., the seminal tubules are formed, those at the hilar region showing a small lumen. Varying amounts of cortex persist at the crest. Figure 2 shows this condition in a three-month old individual (105 mm.). The cortex is reduced to a small lobe and contains germ cells of residual gonial type, usually not more than six in any section. A few primary spermatogonia with polymorphic nuclei are scattered through the center of the testis.

The gonads of females at this age and length are usually much larger than those of the males and in sections show growing ovocytes and large secondary ovarian cavities (ovarial sacs). Figure 3, a female, 105 mm. long, three months old, shows this condition.

In metamorphosing males of four months, average length 135 mm., the seminal tubules often contain spermatocytes in the different stages of chromosome conjugation (Figure 4). Varying amounts of cortex are still present at the crest containing germ cells of gonial type which show signs of degeneration. The degeneration of the cortex is in progress in animals preserved at five months (average length 160 mm., and completely metamorphosed). In cases which show growing ovocytes, the cortex forms a distinct lobe, being separated from the testis proper by a deep constriction.

The degree of development and the extent of the cortex vary so much that one may classify the males of this group as shown in Table I

PLATE I—EXPLANATION OF FIGURES

- Figure 1. *Ambystoma tigrinum*, larva, 32 mm., 4 weeks. Room temperature. Cross section through center portion of differentiating sex gland. X 110.
- Figure 2. *Ambystoma tigrinum*, metamorphosing, 105 mm., 3 months. Room temperature. Lobe of cortex at the crest. Seminiferous tubules near hilar portion with small lumina. Undifferentiated gonidia in rete. X 55.
- Figure 3. *Ambystoma tigrinum*, metamorphosing, 105 mm., 3 months. Room temperature. Well formed ovarial sac and many growing ovocytes. X 55.
- Figure 4. *Ambystoma tigrinum*, with small gill stumps, 140 mm., 4 months. Room temperature. Small amount of cortex still persists at crest; spermatocytes. X 55.
- Figure 5. *Ambystoma tigrinum*. Well developed cortex nearly separated from testicular portion by constriction. X 110.
- Figure 6. *Ambystoma tigrinum*, larva, 59 mm., 8 months. Cold series. Ovocytes scattered among spermatogonia. Well developed cortex. X 110.
- Figure 7. *Ambystoma tigrinum*, incompletely metamorphosed, 138 mm., 13 months. Cold series. Extra regional gonidia in rete and vas efferens. X 110.
- Figure 8. *Ambystoma tigrinum*, larva, 59 mm., 8 months. Cold series. Cortex nearly sterile. Ovogonia, few ovocytes present. X 110.
- Figure 9. *Ambystoma tigrinum*, larva, 60 mm., 8 months. Cold series. Poorly developed ovarial sac. X 110.



Table I—Cortical Development in the Male Gonads of *Ambystoma tigrinum* of Iowa.

- Class O — No trace of ovarian cortex.
- Class I — Degenerated cortex.
- Class II — Single row of gonidia in cortex.
- Class III — Larger numbers of cortical gonidia.
- Class IV — Cortex with ovocytes.

| | O | I | II | III | IV | Total |
|------------------------|---|---|----|-----|----|-------|
| Larvae at 13° C. | — | — | 1 | 7 | 34 | 42 |
| Larvae at 22° C. | — | 1 | 1 | 21 | 8 | 31 |
| Metamorphosed (22° C.) | — | 3 | 6 | 20 | 3 | 32 |

One of the cases of marked hermaphroditism is shown in Figure 5. It is of added interest because the ovarian lobe with its large ovocytes is not attached at the extreme distal surface of the testis, but nearer the hilum.

More *tigrinum* eggs were received from Minnesota, and from near Chicago. When reared it was found that the manner of development and differentiation was the same as that of the differentiated race of Iowa City, described above. The combined groups consist of 188 females + 152 males.

b. Low temperature series

The *tigrinum* subjected to cold temperatures throughout their period of larval development had a high mortality rate. Only 46, or 23 per cent of the 1931 *tigrinum* reached the stage of differentiation. Of this group, one metamorphosed in May, 1932, or 13.5 months after the eggs were placed in the cold temperature. This individual was 114 mm. long. None of the remaining animals showed signs of metamorphosis, so all were preserved on June 1. The average length of these animals at this time was 109 mm. Room temperature animals of this length were already showing loss of gills. The following year more eggs were collected from the same vicinity and 150 of these subjected to the same temperature as the 1931 cold series. Of this group, one metamorphosed at 9.5 months. The rest were preserved in March, or nearly a year from the time the eggs were collected. At this time a few were beginning to show reduction of gills. The average length was 104 mm. The mortality rate of this second series was not very different from that of the preceding year. Thirty-nine, or 26 per cent, reached the stage of sex differentiation. Taken together, these two lots yielded 42 males + 43 females.

All males of the cold series show cortex present. It can be seen from Table I that more cortex is found in the cold series than in corresponding stages of the room temperature series.

The gonads of the individuals of the low temperature series are much smaller than those of equal-sized individuals kept at room temperature. The fat bodies are also small, in some cases barely visible. A few are of fair size, but in no case equal to those of the room temperature series. Differentiation in the cold series is also retarded. In room temperature individuals of 30-35 mm., and 4-5 weeks old the gonads show early phases of sex differentiation. In the cold series this differentiation occurs relatively later.

A male of 59 mm., 8 months old, and not metamorphosed shows a crest of ovarian cortex along the entire length of the gonad. Sections of this gonad do not appear very different from those of room temperature individuals of only 35 mm. In Figure 6 a section near the cephalic end of the gonad, oocytes in pachytene stage can be seen not only in the cortex but also scattered among spermatogonia of the medullary region. These oocytes obviously are carried by the sex cords and will no doubt degenerate later. Sections through the same gonad near its center show medullary germ cells of the primary spermatogonia type, while the cortex contains oocytes up to the pachytene stage.

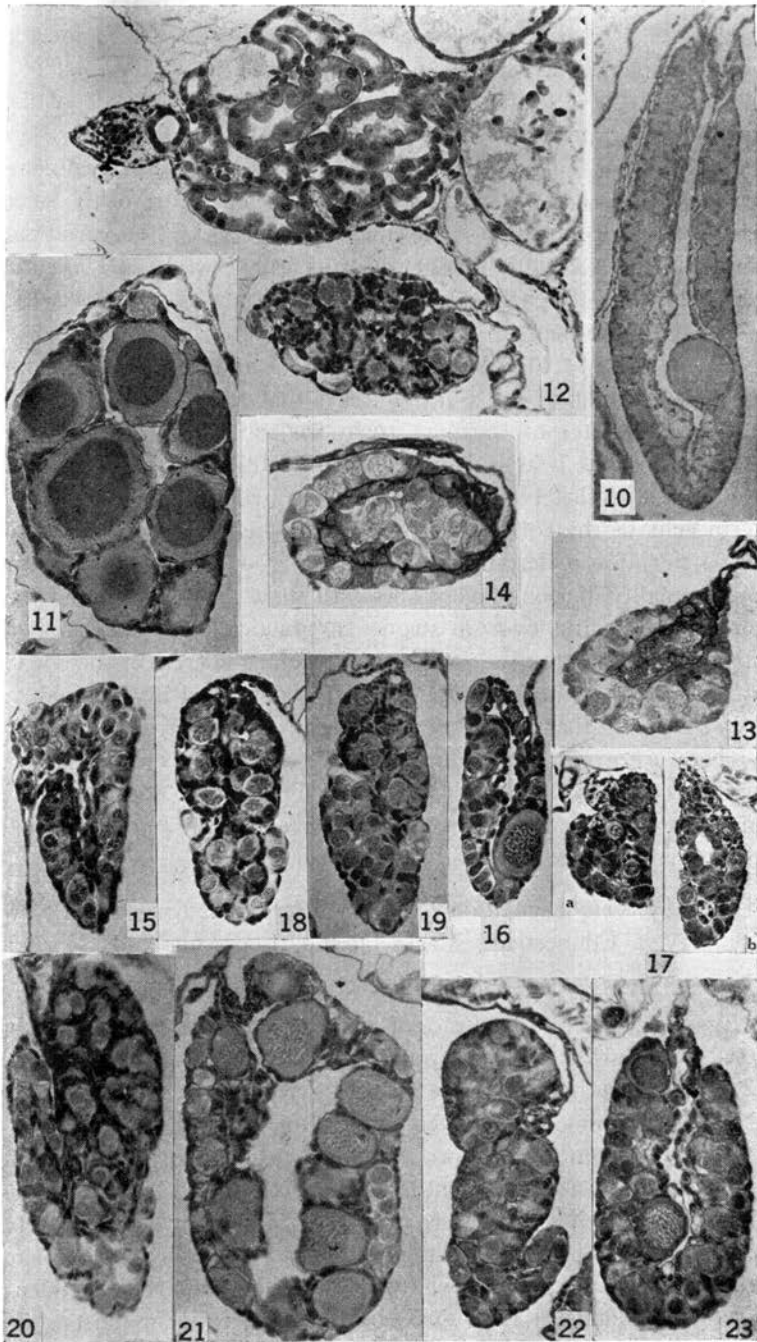
The largest male of the low temperature series was 138 mm., 13 months old, and still not completely metamorphosed (Figure 7). This individual, however, is not any further developed sexually than room temperature individuals of 100 mm., and three months of age. The cortex forms a crest which extends practically the entire length of the gonad, only a few sections of one gonad being without germ cells in the cortex. These cortical cells are for the most part in the pachytene stage or of the primordial type with lobulate nuclei. Sex cord formation at this end of the gonad is still going on. The ectopic (hilar) gonidia in the rete and vas efferens probably would have degenerated later.

It is interesting to note that while in room temperature the gonads are composed entirely of germinal tissues, individuals of the cold series show large amounts of sterile stroma in both ovaries and testes. For example, Figure 8 shows a section of an ovary of a 59 mm. long individual of 8 months. This individual shows a larger ovarian sac than is typical of this series, but no growing oocytes appear in the entire ovary and most of the germ cells are primary ovogonia, or are oocytes of the early synaptene stage.

A female, 60 mm. and 8 months old shows a well developed cortex with oocytes in nests of 2, 4, or 8. The secondary gonadic cavities (ovarian sacs), which in room temperature females of this stage are well formed and inflated, in these cold temperature

PLATE II

- Figure 10. *Ambystoma tigrinum*, gill stumps, 125 mm., 11 months. Cold series. Small ovarial sac. Few growing ovocytes. X 55.
- Figure 11. *Ambystoma maculatum*, metamorphosed, 46 mm., 4 months. Room temperature. X 110.
- Figure 12. *Ambystoma maculatum*. Male (differentiated race), beginning of metamorphosis. Room temperature. Shows a partial rim with primordial gonia. (Comp. Figure 2.) X 110.
- Figure 13. *Ambystoma maculatum*, metamorphosed, 49 mm., 5 months. Room temperature. Cross section near caudal end of gonad. Ovocytes in cortex. Few spermatogonia in testicular portion. X 110.
- Figure 14. Same animal as Fig. 13. Near center of gonad. Poor development of medullary cords. Well developed cortex. X 110.
- Figure 15. *Ambystoma maculatum*. Small gill stumps. 46 mm., 8 months. Cold series. Small ovarial cavity and lack of growing ovocytes. X 110.
- Figure 16. *Ambystoma maculatum* (semi-differentiated race) larva, 29 mm., about 2.5 months. Room temperature. X 110.
- Figure 17. *Ambystoma maculatum* (semi-differentiated race) larva, 23 mm., 2 months. Room temperature. Left gonad (b) still shows the female phase with ovarial cavity characteristic of this race. X 110.
- Figure 18. *Ambystoma maculatum* (semi-differentiated race) metamorphosed, 35 mm., 3 months. Room temperature. X 110.
- Figure 19. *Ambystoma maculatum* (semi-differentiated race) metamorphosing. Room temperature. X 110.
- Figure 20. *Ambystoma maculatum* (semi-differentiated race) metamorphosed, 40 mm., 4 months. Room temperature. X 110.
- Figure 21. *Ambystoma maculatum* (semi-differentiated race), metamorphosed, 45 mm., 4 months. Room temperature. X 110.
- Figure 22. *Ambystoma maculatum* (semi-differentiated race) metamorphosed, 49 mm., 10 months. Cold temperature. X 110.
- Figure 23. *Ambystoma maculatum* (semi-differentiated race) nearly metamorphosed, 50 mm., 11 months. Cold temperature. X 110.



animals are not at all or only poorly developed (Figure 9). Instead, the medullary cords are compact and in many instances include primary gonidia which show signs of degeneration.

Figure 10 is a section of an ovary of the largest female of the lower temperature series (125 mm. long, 11 months old). This female still had gill stumps left. The ovaries are small when compared with those of sister animals in room temperature of the same length or developmental stage (metamorphosis). The ovarian sac shown in this section is small and forms a narrow cleft. The germ cells are still of the early ovocyte stage with a few growing ovocytes—seldom more than two in any section.

Humphrey (5), describing conditions of ambisexuality in *Ambystoma maculatum* and *tigrinum*, says that the "bisexual" condition in the testes of *tigrinum* is so frequent as to warrant the assumption that it is a normal phase of testicular development. He also states that two-thirds of *tigrinum* males examined showed germ cells of the cortex in first ovocyte stages. In our material all 63 *tigrinum* males from Iowa kept at room temperature show ambisexuality, but only about one-sixth show the germ cells of the cortex in the first ovocyte stage. In practically all the cases of this group the cortex forms a distinct lobe at the crest of the gonad rather than an "antimesorchial layer" as described by Humphrey. It would seem that Humphrey's material comes nearer to our low temperature group with more extensive cortical formations and a high percentage of ovocyte production. This author does not make any statement, however, relative to the temperature under which his animals were raised.

II. *Ambystoma maculatum* (Shaw), differentiated race from New Haven, Connecticut, Massachusetts, Louisiana and Georgia.

a. Room temperature series

Eggs of a differentiated race of *Ambystoma maculatum* (Witschi 10, 15, 18) were received from New Haven, Connecticut, April 12, 1931. Two hundred were placed in room temperature (22-28° C.) and two hundred at a temperature of $13 \pm 2^\circ$ C.

The first room temperature individual to metamorphose was 51 mm. long and was three months old. Practically all had metamorphosed by September and were preserved (5 months).

The individuals at room temperature show an early and complete sex differentiation and are relatively free of hermaphrodite tendencies. The gonads of individuals of 18-22 mm. are in most cases still indifferent. Those of 30-35 mm. are differentiating. All

individuals of ten weeks and an average length of 43 mm. are completely differentiated although Burns (1) states that differentiation is first noticeable at 10-12 weeks. It is likely that this investigator had kept his animals at a room temperature lower than ours. Formation of seminal tubules begins in individuals of 55-60 mm. and 20-25 weeks of age. About the time of metamorphosis, only four cases of extreme ambisexuality similar to those pictured by Humphrey (5, Figures 6 and 8) were found; two were still at the larval stage.

The result of the room temperature series was 65 females + 72 males which comes close to the expected 1:1 ratio for a differentiated race. Burns (1) and Witschi (18) have described sex differentiation in *Ambystoma maculatum* and my finding substantiates theirs in the main features.

The room temperature series of the New Haven race shows less ambisexuality in males than was found in the series of *Ambystoma tigrinum* previously described. When present, the cortex in *Ambystoma maculatum* forms a thin shell around the testis, rather than an antimesorchial lobe as in the *Ambystoma tigrinum*.

Table II shows frequency and extent of ambisexuality found in both larval and completely metamorphosed males.

Table II—Cortical Development in Males of *Ambystoma maculatum* of New Haven, Louisiana, and Massachusetts (Classes as in Table I).

| | O | I | II | III | IV | Total |
|----------------------|----|----|----|-----|----|-------|
| Larvae 13° C. | — | — | — | 2 | 1 | 3 |
| Metamorphosed 13° C. | 7 | 5 | 5 | — | — | 17 |
| Larvae 22° C. | 2 | 8 | — | 1 | 2 | 13 |
| Metamorphosed 22° C. | 29 | 74 | 14 | 2 | 4 | 123 |

The room temperature series of *Ambystoma maculatum* differentiates in a similar manner as the differentiated frog races (Witschi 10, 11, 13, 14, 15). The condition attained by females and males about the time of metamorphosis is illustrated by Figures 11 and 12. Comparison with Figure 2 brings out the fact that testicular differentiation in *A. maculatum* lags behind that in *A. tigrinum*.

Only one room temperature individual out of the males shows an extensive cortex. This animal of 49 mm. and 5 months was fully metamorphosed. The testis shows an extensive ovarian cortex over its full length. Figure 13, taken through the caudal part, appears more ovarial than testicular. Only one germ cell is visible in the medulla, while the cortex contains numerous nests of

ovocytes. Figure 14, a section through the central part of the same gonad, shows a cortex with a single layer of gonia and ovocytes while the medulla is crowded with spermatogonia. The interstitial tissue between the testis proper and the ovarian cortex forms a thin membrane which takes a heavy Congo red stain, making the cortex appear sharply delimited from the testicular portion. The other five males falling in class four show also a single layered shell of ovarian cortex containing mainly primordial gonia with polymorphic nuclei. A few germ cells are in degeneration and in all three cases of growing ovocytes (auxocytes) are found. One larval individual shows a number of ovocytes (leptotene stage) scattered throughout the medulla of the testis.

b. Low temperature series

In this group the first individual metamorphosed at nine months and was 55 mm. long. The last one metamorphosed at thirteen months. The mortality rate was much higher than in the *Ambystoma tigrinum* at the same temperature; most of the individuals died before the stage of differentiation. The result of this series was 20 females + 20 males.

The females of this series show in general the same condition as found in the corresponding tigrinum series; small ovarian cavities, late differentiation and development, columnar epithelial cells, and few growing egg cells. Nearly every ovary of this series shows some abnormal condition such as the presence of adipose tissue in the ovarian sac. Sometimes the ovaries are reduced to mere strips of cortex over the edge of the fat body. The fat bodies are much larger than in the tigrinum series. The ovarian cavities are small, slit-like openings. There is a remarkable scarcity or absence of growing ovocytes (Figure 15). It is of interest to compare this group with the females of the room temperature group of equal size which are four months of age and have metamorphosed (Figure 11).

The males also show the retarding influence of low temperature. Structurally, their gonads show no significant differences from those of the controls in room temperature, nor is the amount of cortical tissue increased (see Table II).

III. *Ambystoma maculatum* (Shaw), semi-differentiated race from Arkansas.

Eggs were received from Imboden, Arkansas in the spring of several successive years. Each time some were placed in room temperature and an equal number in cold temperature (13° C.).

a. Room temperature series

The individuals of the room temperature group taken together consist of 146 females and 111 males. Differentiation began in larval individuals of 22-29 mm. With the appearance of secondary gonad cavities and formation of ovocytes in the cortex, females of 29 mm. and slightly older show early ovocytes as well as a few growing ovocytes (auxocytes) (Figure 16). The gonads of males of this same age and length in many cases appear like young ovaries with narrow cleft-like cavities in the center. In some, however, testicular transformation has started. The walls of the ovarian sacs are thickened and thus are transforming into the rete apparatus. The germ cells are migrating into this medulla which progressively becomes more prominent. Figure 17 shows this condition in a male of 23 mm.

In males just metamorphosed varying degrees of ambisexuality and hermaphroditism are observed. The cross section through the testis of a recently metamorphosed male (35 mm.) which is represented in Figure 18 is typical of many individuals of this length. In males of four months and over, it was often found that more germ cells were present in the cortex than in the testis proper. In 39, or 35 per cent of the cases, ovocytes were present in the cortex while the germ cells in the medulla were still of the primary spermatogonial type.

Table III shows frequency and development of cortex in individuals of the room temperature and cold temperature series.

Table III—*Cortical Development in the Males of Ambystoma maculatum, semi-differentiated race of Arkansas*

| | O | I | II | III | IV | Total |
|----------------------|---|---|----|-----|----|-------|
| Larvae 13° C. | — | 1 | — | 3 | 1 | 5 |
| Metamorphosed 13° C. | 2 | 4 | 2 | 4 | 3 | 15 |
| Larvae 22° C. | — | — | — | 8 | 7 | 15 |
| Metamorphosed 22° C. | — | — | — | 36 | 60 | 96 |

Witschi (18) has described this hermaphroditic condition in the Ozark race and says: "At the time of metamorphosis the testicular component usually is well-organized, while the cortex becomes relatively less important." In general, my findings corroborate his. However, at the time of metamorphosis or after, in 9 out of 60 cases of Class IV (15 per cent) the ovarian cortex is the more conspicuous part of the gonads (Figure 19), forming more than half of the total volume of the sex gland.

Figure 20 shows a metamorphosed individual, 40 mm. long and

four months old. Here the cortex at the crest shows many germ cells in degeneration. No doubt they will all undergo degeneration, the epithelium becoming reduced to a single peritoneal covering. The cortex is rather sharply delimited from the testicular portion. All germ cells of the testicular portion are of the spermatogonial type.

The females of this series develop like those of New Haven race. Differentiation takes place at about the same age and growth and ovocyte formation proceed at a similar, possibly slightly slower, rate (compare Figure 21 with Figure 11).

b. Low temperature series

In the cold series 32 per cent reached the stage of metamorphosis. At six months a few larvae were beginning to show loss of gills but only one had completely metamorphosed at nine months. This individual was 50 mm. long. The remainder were preserved the following March; a little over a year after the beginning of the experiment. They still had gill stumps present. The average length at this time was 60 mm.

In the males the cortex is less extensive than in the room temperature series. Figure 22 refers to the specimen which had the largest amount of cortex found in any of the 17 males of the cold series. At the cephalic and caudal ends of the gonads the cortex consisted of but a thin peritoneal covering, the cells of which had

Table IV — Sex Ratios of Ambystoma

| Room temperature 22° C. | | | | |
|---|--------|------|-------|----------|
| Species | Female | Male | Total | Male % |
| Tigrinum (differentiated) from Iowa City, Chicago, Minnesota | 188 | 152 | 340 | 45 ± 1.8 |
| Maculatum (differentiated) from New Haven, New Orleans, Massachusetts | 176 | 228 | 404 | 56 ± 1.4 |
| Maculatum (semi-differentiated) from Imboden, Ark. | 161 | 125 | 286 | 44 ± 1.6 |
| Total | 525 | 505 | 1030 | 49 ± 1.5 |
| Low temperature 13° C. | | | | |
| Tigrinum (differentiated) from Iowa City | 43 | 42 | 85 | 49 ± 3.7 |
| Maculatum (differentiated) from New Haven, Massachusetts | 20 | 20 | 40 | 50 ± 5.3 |
| Maculatum (semi-differentiated) from Arkansas | 31 | 20 | 51 | 39 ± 4.7 |
| Total | 94 | 82 | 176 | 47 ± 2.5 |

All groups come fairly close to the expected 1:1 sex ratio with the exception of the Arkansas variety of *A. maculatum*.

large columnar darkly staining nuclei. The center sections, however, show a small crest of cortex with germ cells of early ovocyte type. The males of this series show more spermatogonia in the testicular portion of the gonads than were found in the room temperature series of the same race.

Figure 23 shows a typical female 50 mm. long and eleven months old and nearly metamorphosed. Even in center sections few growing ovocytes are present, usually not more than three in any section. The ovarian cavity is narrow. Room temperature animals of similar length (48 mm. long but only four months of age) show large numbers of growing ovocytes (Figure 21) and large ovarian sacs.

DISCUSSION

Ambisexuality in Ambystoma

The term ambisexuality is primarily used to indicate the co-existence of cortical and medullary primordia which is generally observed in amphibian gonads before sex differentiation. However, it applies also to all cases in which the primordium of the opposite sex persists after sex differentiation. Thus the testes of the salamanders remain ambisexual as long as they retain cortical rudiments with undifferentiated gonia. It is not as easy to decide morphologically how long the ovary retains its ambisexuality. Medullary rudiments are permanently retained. It is not yet clear how long they keep the potentiality of primordia of testicular development. No cases of transformation of adult female salamanders into males have yet been reported, though the morphological basis for such a change seems to be present in every ovary.

The term hermaphrodite has been used to describe every possible combination of male and female structures occurring in one individual. In a very restricted sense, hermaphroditism applies only to the situation in which the same individual produces mature eggs and sperms simultaneously or successively. In the case of so-called juvenile rudimentary hermaphroditism we have a condition in which during the early development an ovotestis produces both ovocytes and spermatogonia, but later changes over into a purely testicular or ovarian sex gland.

In some cases of *Ambystoma maculatum* there is normally little or no tendency toward hermaphroditism. The males show early and complete sex differentiation (so-called differentiated race). Males of the same species but from a different local variety all first pass through a female phase and therefore are juvenile hermaphrodites.

At the outset of sex differentiation their medullary cords possess central cavities, the rudimentary ovarian sacs. At the same time a typical ovarian cortex with ovocytes forms which persists even after testicular differentiation has started during the second half of the larval period. This is the so-called semi-differentiated race of *A. maculatum* of the Ozarks.

This is in agreement with the work of numerous investigators since the initial work on frogs by Pflüger. Further work by Hertwig (4) and Witschi (10, 11, 12) shows hermaphrodites of protogynous type common in so-called semi- and undifferentiated races of frogs.

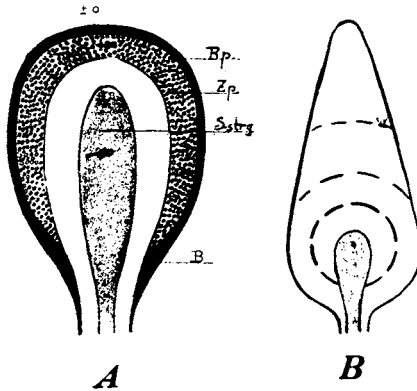
The neutral zone of the cortex and progress of inductive sex differentiation

When sections of *Ambystoma tigrinum* males are studied it is found that 82 per cent of the room temperature series have cortical rudiments located at the region of the crest. In most cases all gonads are triangular in shape. In differentiating males seminiferous tubules are first formed at the hilar region and develop successively toward the crest. Near metamorphosis the ampullae nearest the hilar region have well developed lumina while those in the center of the gonad are solid buds and in the region of the crest sex cords are still in evidence which carry undifferentiated gonidia from the germinal epithelium into the testicular portion (Figure 2). This last feature is somewhat different from that observed in Triton (or Selachians) where the cortical germinal epithelium at the crest of the gonad exists throughout lifetime and continually proliferates (Witschi 19). In the tiger salamander, the proliferation of sex cords stops about metamorphosis time. For awhile there remains, however, the cortical crest with indifferent primordial gonidia. Finally, these disappear by degeneration and sloughing off.

Figure B (Text) is an attempt to show in a graphic way how this developmental progress may be interpreted on the assumption that an inductive center is localized in the medulla near the hilum. The medulla is the seat of the male induction which spreads through the tissue of the gonad with falling gradients expressed by the dotted circle. Parts nearest to the inductor are the most advanced in testicular differentiation because they have been exposed to the inductive influence first and most intensely. The rather short range of positive induction is in sharp contrast with the more general spreading of inhibition of cortical differentiation (Witschi 16, 18, 19).

Occasionally some tigrinum gonads show two crests of cortex; apparently their formation is due to abnormal pressure which has deformed the young gonad. Such occurrences suggest that the "crest" is not a pre-established differentiation but rather that any part of the primordial cortex can form a crest and retain primordial germ cells if it is far enough removed from the inductive center. In *Ambystoma maculatum* (differentiated race) the young testis is more rounded (Figure 12). In 72 per cent cortical rudiments are present though they are not concentrated at the crest as in the *Ambystoma tigrinum*. If a few cortical gonidia are left, they can be found just as well in the lateral or hilar parts of the peritoneal covering of the gonad. Occasionally, however, one finds deformed testes that show cross sections very much like those typical for tigrinum. In such cases one finds again the undifferentiated gonidia preferably at the crests, which adds additional evidence in favor of the inductive theory.

It is also of particular interest that in ovaries of both species one observes with great regularity the occurrence of some primordial gonidia along the hilar ends of the cortex. This indifferent zone is especially pronounced toward the caudal end of the ovary.



EXPLANATION OF TEXT FIGURES.

Figure A. Diagram of anuran gonad primordium. B. hilar part of neutral zone. Zp. Inductive part of cortex. S. strg. Inductive part of medulla. Rp. Neutral zone. + 20, — 10, \pm 10 represent induction values of the designated parts. (After Witschi '14.)

Figure B. Diagram of urodele gonad primordium illustrating how induction spreads from medulla in falling gradients, expressed by the dotted lines. The medullary inductor has a relatively short range and therefore allows the crest in *A. tigrinum* to retain primordial gonidia up to metamorphosis.

The same condition has been described by Witschi (10, 11) as characteristic of gonads of frogs. He has pointed out that in cases of sex reversal and in the so-called indirect differentiation of testis in races with hermaphrodite tendencies this reservoir of primordial gonia becomes the main supply for the formation of sex cords and spermatogonia (Figure A, text).

Work of Humphrey (6) and Burns (2) has given ample proof that these hilar rims can play exactly the same role in *Ambystoma*. During the early stages of male differentiation of semi-differentiated maculatum salamanders, we find primordial gonia almost evenly distributed the full extent of the peritoneal covering. When delayed testicular development follows, the germ cells from about the hilar halves of the cortex move into the sex cords while those of the crestal halves transform into oocytes, with the exception of some occasional gonia at the extreme periphery which remain undifferentiated.

All these observations bring out the general fact that the extreme periphery of the cortex constitutes a "neutral zone." This neutral zone is not of uniform thickness. In many cases in *tigrinum* males near metamorphosis, it is almost entirely restricted to the crest of the gonad. In females of all amphibians it is restricted to the so-called "germ patches" (Witschi 15) and the hilar rim of the cortex. Since the germ cells of this "neutral zone" remain unaffected by differentiating inductive influences, they constitute the natural reservoir out of which amphibian females are supplied with large numbers of yearly maturing germ cells. The neutral zone loses, however, the function of a "germinal epithelium" in the males of most amphibians and of all higher vertebrates already during the early embryonic development, when sex cords cease to form.

It seems that in higher vertebrates, especially in mammals, even in the female sex, the neutral zone entirely disappears at an early stage of life. No new oocytes can be formed thereafter. In males a new type of "germinal epithelium" is formed within the seminal tubules. In the testes of *A. tigrinum* one finds regularly spermatogonia of primordial type near the place where they attach to the rete testis. The translocation of the primordial germinal elements from the neutral zone of the cortex to the medulla of the gonad constitutes in fact the most characteristic features of early testicular differentiation. No instances are known, so far, of a reverse translocation. In this sense no instances of male to female reversal have been observed so far.

Developmental factors operative in sex differentiation

There are cases where in the process of the development of the male gonads of *tigrinum* a part of the gonad is partially constricted off from the testicular region (Figure 5). In these atypical cases the cortex reaches its greatest development. It appears that this constriction acts as a barrier to the stimulating effect of the medulla and allows the cortex in this isolated portion to develop. In other words, the cortex has been relieved from the medulla antagonist. In other cases pertaining to the differentiated race of *maculatum* (Figures 13 and 14) there is a lack of development of the medullary cords. This, again, means a lowering of the medulla control and allows the cortex to become well developed. Whenever the inhibiting action of the medulla is decreased, the cortex is always ready to respond with compensatory development.

In the room temperature series of *Ambystoma tigrinum* the incidence of hermaphroditic features in males is 17 per cent; in the differentiated race of *A. maculatum* it is only 4 per cent while in the semi-differentiated race it is 60 per cent. The study brings out the fact that in those normal control animals of different races of the species studied, atypical cases of hermaphroditism appear with certain regularity and in about the same ratio as they appear under many experimental conditions. Food could not be a factor favoring these atypical cases since all animals were fed the same diet and with equal regularity. The occurrence of hermaphroditism is in no way related to size variation.

Since slight variations in temperature can not be easily controlled, the temperature factor is tested in a second set by lowering the temperature considerably. Tables I, II and III show that in the cold series hermaphroditism was increased in *tigrinum* while in the races of *maculatum* the tendency was not consistently changed. The condition in *maculatum* may mean that the temperature was not lowered below the critical point and this would further mean that low temperature is not a causal factor in the development of hermaphroditism in this species. Mortality at the temperature employed makes it impossible to decrease it further. Temperature factors of the cortex and medulla in this species are identical within the tested range and do not spread apart as they apparently do in *A. tigrinum*. Witschi (10, 11) brought out the fact that cold temperature had a feminizing influence on differentiated races of frogs. The males of these races, if kept in cold, pass through a female phase similar to that of the semi-differentiated race. Ap-

parently cold retards the medulla development temporarily and allows the cortex to develop. This has been confirmed by Piquet (8).

In all the females of the cold series the ovarian sacs are small; this does not mean a shift in male direction though it is a striking fact that also the growth of ovocytes (auxocytes) is retarded.

Genetical factors

The fact that animals with atypical hermaphroditism appear with certain regularity in the differentiated races of ambystoma under normal as well as under experimental conditions and that the tendency is greater in some races than others of the same species makes it appear to be an hereditary trait and therefore must be given a genetical interpretation. Breeding experiments of Hertwig (4) and Witschi (10, 11, 14, 15) led the latter to suggest that the sex genes might be aggregates of smaller units which can be called sub-genes (Witschi 17). If one assumes that these sub-genes are distributed along a carrier chromosome, it would follow that homologous elements may be exchanged by crossing over. Plus and minus variations of sex determining sub-genes would have a chance, therefore, to combine in many different ways. In some male zygotes the combinations should be so that the male determining factors are relatively low and the female determining factors relatively high. These combinations would account for the males which under normal and experimental conditions exhibit decidedly hermaphroditic tendencies.

The importance of genetical work on amphibians arises from the fact that the mechanism of hereditary sex determination is here obviously in a state of becoming established.

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