Reciprocal Transplantations of Testicular Grafts in Two Salamander Species

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Thanks to the previous works issued from our institute it has been well established that Hynobius retardatus in Sapporo is the hermaphrodite (semidifferentiated) species, while H. lichenatus in Aomori is the gono-choristic (differentiated) species. It may be probable that the hormonal intensity is different in these two species because of the variation in their genetical constitutions. Firstly several attempts had been done to produce the hybrids between the two species, but were all failed. By artificial fertilization the eggs of these species developed to the embryos, but they were all abnormal in form and did not make the swimming larvae. Secondly embryos of the two salamanders were grafted together in the left to right position. They were reared to the stage of metamorphosis and their gonads were examined (Uchida & Hanaoka, 1941). The results were as in the following. In the homogenous pairs of H. retardatus × H. retardatus, the gonads of homosexual twins seemed to be not affected each other, while the gonads of heterosexual twins were mostly normal but some ovaries appeared to be modified. In the homogenous pairs of H. lichenatus × H. lichenatus, sex distribution was 4♀♀ + 6♀♂ + 23♂♂ = 33. The gonads of ♀♀ pairs were normal in structure and were more advanced in differentiation than in the gonads of the controls. In the heterosexual twins the testes were normal but the ovarian development was always severely arrested. In ♂♂ pairs the testes were sometimes both normal but sometimes the testis of one member was very small and beared a large number of degenerating germ cells. Judging from the excess number of this combination, the small testes just mentioned might have been transformed from female gonads. In the heterogenous pairs of H. retardatus × H. lichenatus, the sex distribution was as follows; 10♀♂ + 8 (lichenatus ♂ + retardatus ♀) + 7 (lichenatus ♀ + retardatus ♂) + 30♂♂ = 55. In the ♀♀ pairs the ovaries were all normal. In the ♂♂ pairs, the differentiation of both testes was mostly normal, but in a few pairs, the testis of one component was nearly sterile. In the normal pairs the testes of H. retardatus alone were subject to the elimination of germ cells. In 4♂♂ pairs, the gonads of retardatus had
some female traits. It is possible that they were in the process of transformation from female to male. Among the heterosexual twins, combinations of lichenatus ♀ × retardatus ♂ gave sometimes no effects on the gonads of the both members but sometimes slight effects on both of them. In the pairs of lichenatus ♂ × retardatus ♀, the gonads of the latter were found to be always arrested in differentiation and sometimes became sterile, while the gonads of the former were all normal. From this experiment it can be confirmed that the male of the gonochoristic (differentiated) species inhibits the ovarial differentiation in the female twin of its own species or of the hermaphrodite (semidifferentiated) species, whereas the male of the hermaphrodite species never fully suppresses ovarial differentiation of the female twin of its own species or of the gonochoristic species. It was therefore concluded that the testicular medulla of H. retardatus (hermaphrodite species) is less antagonistic to the cortex than the medulla of H. lichenatus (gonochoristic species).

On the other hand, the writer and his collaborator have studied the sex-differentiation of the two salamanders by the homoiotransplantation of testicular grafts respectively (1937, 1939 & 1942). After these experiments the reciprocal transplantations of testicular pieces have been here carried out in the two salamander species. A part of the expense of this study has been defrayed from the Scientific Research Expenditure of the Department of Education. Before proceeding further, the writer must express sincere gratitude to Dr. K. Hanaoka for several helps in the preparation of this paper.

Experiment I

Gonads of Hynobius retardatus affected by the testicular grafts of Hynobius lichenatus.

The larvae used in this experiment were approximately 20 mm in the whole length and had fore limbs, 1.4 mm long, and buds of the hinder limbs. In these larvae the gonads were not yet developed. The first autopsy occurred 3 weeks after the operation. Thenceforward the autopsy was taken place weekly. Such repeating 50 healthy specimens were examined.

The larvae of the first group were killed 3 and 4 weeks after the operation. They were 7 in number and measured 28–32 mm in the whole length. They had still primitive gonads in which elimination of cortical germ cells is very prevailed, thence the germ cells have become very few in number. The germ cells are generally separately situated on the cortical layer but rarely three or four ones are grouped in the distal portion as in the case of the homoiotransplantation of the testicular graft in Hynobius retardatus. No medullary immigration of germ cells was observable.
The larvae belonging to the second group were examined 5 weeks after the operation. They are 4 in number and 34–35 mm in length. The gonads of these larvae are larger than those of the first series but nearly similar in form to the latter. Medullary immigration is slightly observable. The germ cells are sparsely distributed and are found nearly on the cortical layer. The figure (Fig. 1) shows a section through the posterior portion of one of these gonads, which is the most masculinized among them. The medullary portion is compactly packed with two germ cells and interstitial cells surrounding the former. However, three germ cells lying on the cortical layer are about to be eliminated. In sections through the middle portion of this gonad the medullary portion is solid with interstitial cells but has no germ cells. Sections through the anterior portion of the same gonad are clearly different from those of the posterior portion. They display the free-martin gonads as shown in the homoio-transplantation, with germ cells aggregated on the terminal free end.

The larvae belonging to the third group survived 6–7 weeks after the operation and were 41–48 mm in the whole length. Eight specimens were secured. The normal larvae in this stage are in the ovarian stage, with a distinct ovarian cavity. The gonads of these experimental animals are masculinized or of affected female. Out of them 5 are of the male type and 3 others are of the female. The male gonads are hardly different from the normal gonads but seem to contain rather small number of germ cells; the germ cells are probably eliminated violently by the effect of the testicular implantation, because some gonads among these male gonads have a few germ cells lying on the cortical layer which are on the spot of elimination. In a male gonad, in which the germ cells are mostly distributed in the distal half, a remnant of the ovarian cavity is distinctly observable. The gonad expressed by Figs. 2–3 is very interesting; the anterior section of the gonad is typically of the male type as shown in Fig. 2, with marked germ cells surrounded by rete cells, though the cortical layer is partially present; but in the posterior sections the gonad displays an affected female gonad. As is expressed in Fig. 3, the germ cells, which seem to be inactive, are arranged in a sparse row on the distal margin and are just in elimination. In this region the cortical and medullary portions are distinctly lined. The medullary portion is not compact and does not display the definite male feature. The three female gonads belonging to this group are all affected; their ovarian cavity is usually loosely packed with rete cells and the cortical germ cells are largely degenerated in situ and survive as a mere sparse row as shown in Fig. 4. Sparity of germ cells seems to be rather due to the degeneration than the elimination. The degeneration of the germ cells in the female gonads is a marked character seen in experimental animals through the whole stage.
The fourth group of experimental larvae includes 7 specimens, 45–58 mm in the whole length, which survived 8–9 weeks after the operation. With the exception of one specimen, they were over 50 mm. In the

Figs. 1–10. Gonads of *Hynobius retardatus*. 
control animals the gonads in this stage are nearly sexually differentiated. Among these specimens, 4 are typical males which have well-developed sex-cords. There were found no male gonads of which germ cells are sparsely scattered. The 3 females are all affected and have the ovarian cavity which is partially or entirely packed with rete cells. The cortical germ cells are partially degenerated and partially eliminated, so they are often arranged in a discontinuous row, accompanied with meagre interstitial cells. Two gonads of them just mentioned show two selective diverse phases in sections; in the anterior portion of them the ovarian cavity is completely packed with rete cells containing germ cells (Fig. 5), but in the posterior portion the ovarian cavity is surrounded by the somewhat degenerated cortex (Fig. 6).

The fifth group of the experimental animals contain 8 specimens, ranging in length from 51 mm to 54 mm. They were all half-metamorphosed and fixed after 10 weeks since the operation. The control animals corresponding to this stage were already metamorphosed and completed the sexual differentiation. Out of them, 3 are typical males and 5 are more or less affected females. Two females of them are nearly typical females, though slight elimination and degeneration of germ cells, and reduction of the ovarian cavity are observable. Three other female gonads are characteristic in having a large ovarian cavity which is formed by a rather thin cortical part (Fig. 7). The elements forming the cortical part are mainly germ cells, bearing rather few interstitial cells. In these gonads germ cells are fairly or largely degenerated and are sometimes arranged in a loose row, surrounded by rete cells which are very meagre and inactive (Fig. 8). Though the ovarian cavity is large, the cortical layer is distinctly thin, compared with those of the control females. It is noticeable that in these gonads the cortical elements are more or less degenerated but the ovarian cavity is always clearly open, showing no sign of medullary immigration of rete cells.

The sixth group of the experimental animals contains 16 specimens which were 46–51 mm in length and all metamorphosed. The autopsy occurred in 11–12 weeks after the operation. Out of these specimens 7 are males. They are all normal and well-differentiated. Among the females one has the degenerated ovaries with the large ovarian cavity as described above. The 7 remaining gonads have all the ovarian cavity more or less diminished in size and sometimes packed with rete cells often including sex-cords (Fig. 9). The cortical germ layer of these ovaries is always active and the elimination was scarcely observed in it. The appearance of large oocytes is rather late comparing with the result of homoiotransplantation. They could not be seen in gonads of 11 weeks after the operation but found in all gonads of 12 weeks after the operation.
The ovarian cavity is always narrowed and often filled with oocytes or ova.

Judging from the results the gonads of the experimentals, differing from those of the controls, do not pass through the undifferentiated phase and the ovarian stage. The male gonads are always activated in development and the female gonads are all affected. After the metamorphosis, however, the genetical tendency gradually surpasses the hormonal effect, thence the affected gonads gradually resume the normal female features. When compared the results of this experiment with those of the previous experiment of testicular homoiotransplantation in _H. retardatus_, the following facts were proved. In the homoiotransplantation the elimination and degeneration of germ cells are not so violent, so the gonads bear rather many germ cells which are grouped in the distal portion. In the heterotransplantation the elimination of germ cells is distinctly predominant and the degeneration of female germ cells very frequently occurred. In these affected gonads, however, the phenomena of masculinization are scarcely met with: the ovarian cavity is nearly in all cases spacious, though slight immigration of rete cells has taken place. These features of the affected gonads recall the degenerated gonads of the writer's high temperature experiment (1937) and the deformed gonads effected by testosterone-propionate (see Hanaoka, 1941). The different results by these two experiments seems to be due to the different effects of male hormones produced by _H. retardatus_ and _H. lichenatus_. It will be discussed later on.

**Experiment II**

Gonads of _Hynobius lichenatus_ affected by the testicular grafts of _Hynobius retardatus_.

The larvae used in this experiment were about 20 mm in length and had fore limbs, 1.4 mm long and rudiments of the hinder limbs. They were almost similar in size and development to those of _H. retardatus_ in Experiment I. In these larvae the gonads were scarcely developed. They were at first killed 3 weeks after the operation and thenceforward several specimens were preserved once a week. The larvae examined were 49 in all and slightly smaller than those of _H. retardatus_ in each stage.

The larvae belonging to the first group were examined 3–4 weeks after the operation. They were counted 8 and measured 24–28 mm in the whole length. The gonads of these larvae have always scarce germ cells. The germ cells are sometimes inactive on the cortex and often in the state of elimination, but in some gonads are very active and are located in the medullary portion, taking the form of the primitive male gonads. The gonad expressed by Fig. 11 is of complicated nature; it has
in the distal portion two cortical germ cells which will be eliminated before long and in the proximal part two medullary germ cells.

The larvae of the second group were killed 5–6 weeks after the operation. They were 8 in number and 27–36 mm in length. The gonads of the control animals corresponding to this stage are still in the indifferent stage. In the gonads of the experimental animals are included one primitive gonad, one male gonad, four female gonads and two modified free-martin gonads. The male gonad is yet small and contains small numbers of germ cells in the medulla. It is very peculiar that the modified free-martin gonads as seen in the retardatus-homoiotransplantation are seen in two gonads. Such a gonad has never been observed in the normal gonads of H. lichenatus and in the gonads of H. lichenatus harbouring the testicular grafts of the same species. In the modified free-martin gonad germ cells are aggregerated only in the distal portion of the gonads and active rete cells are proliferated in the proximal part. The modified gonads in this series are all small in size and have a few germ cells only on the peripheral region (Fig. 12). The germ cells in the distal portion are to be eliminated in the future. Out of the four female gonads, two gonads are active, though provided with a narrowed ovarian cavity. A female gonad has the ovarian cavity solid with rete cells. Another female gonad has cortical germ cells more or less in elimination and the ovarian cavity full of rete cells (Fig. 13). It is noticeable that the female gonads retain female features, though slightly affected, in spite of the presence of testicular grafts.

The larvae belonging to the third group were 7–8 weeks old after the operation and 25–38 mm in the whole length. They were counted 10 and all over 30 mm except two exceptional ones. Out of them 5 gonads are of the male type. They are small in size and always contain meagre germ cells in the medulla. In some gonads the elimination of a few inactive cortical germ cells was observable. Some of these gonads have probably regenerated from the modified female gonads. In fact, there is found a modified free-martin gonad which bears several germ cells in mass at the distal end of it, which are probably to be eliminated before long. In this gonad the rete cord proceeds to invade to the medulla but no germ cell is yet found in it (Fig. 14). Besides the gonads above mentioned, there are 4 female gonads which are far larger than the male gonads. They are all modified. These gonads have all fair number of cortical germ cells which are slightly being eliminated. The medullary portion of these gonads is always concentrated with solid rete cells which are very active in proliferation (Figs. 15–16). In these solid gonads, influenced by the testicular grafts, rete cells proliferate in the medullary portion, but on account of weakness of the hormonal effect the elimination of cortical germ cells has not markedly been carried out. The anterior
section of the gonad shown in Fig. 15 bears some resemblance to the modified free-martin gonads of *H. retardatus* but is quite different from the latter in having many active germ cells as shown in Fig. 17. The

Figs. 11-23. Gonads of *Hynobius lichenatus.*
occurrence of rather many cortical germ cells at the distal portion seems to be due to the want of the effective hormone of elimination and of degeneration, produced by the testicular grafts of *H. retardatus*. The anterior section of the gonad expressed by Fig. 16 manifests the advanced stage toward the male gonad, with exuberant ingrowth of active rete cells to the medulla (Fig. 18).

The fourth group is composed of 8 larvae, 27-48 mm in the whole length. They survived 9-10 weeks after the operation. The controls corresponding to this stage are already completely sexually differentiated. Among these specimens, two male gonads are normal, while 6 female gonads are all more or less affected. Out of the six female gonads, three ones are provided with the ovarian cavity, while the ovarian cavity of the others are packed with rete cells. Those with the ovarian cavity are nearly normal female gonads, though the ovarian cavity is always somewhat narrowed and cortical germ cells are slightly eliminated. The gonads with the solid medullary portion have fair numbers of cortical germ cells. In one of them, however, the germ cells are very scarce probably owing to elimination and sparsely arranged in the distal portion. In the anterior portion of this gonad the medulla and cortex are demarcated each other, but in the posterior part the rete cells are loosely distributed and the cortex is barely represented by a few cortical germ cells arranged in a scarce row. Two other female gonads has many active germ cells which are not only thickly arranged in the cortical part but also in the medullary portion wrapped by the rete cells (Fig. 19). In this series of gonads slight elimination is surmised and active medullary immigration is ascertained.

The larvae belonging to the fifth group were killed 11-12 weeks after the operation. They were 8 in number and 25-58 mm at the autopsy. Except 3 ill-developed specimens, all the specimens were over 42 mm and correspond to the controls which completed the sexual differentiation. Out of them 5 gonads are of the male type (Fig. 20). These gonads have always a hyaline part which is nearly bare of germ cells and rete cells. In the three gonads of them the hyaline part is very vast and the active part of the testis is restricted to a locality (Fig. 21). Such a hyaline part is generally seen in degenerated part of testicular graft. The part usually seems to gradually disappear partly on account of the invasion of proliferating cells from the active part and partly by degeneration. It may be probable that these gonads belong to those which have regenerated from the affected female phase. These 3 gonads have several fragments of the cavity in the hyaline part as shown in Fig. 21. Two of the remaining 3 gonads are distinctly of the female feature. These ovaries are almost normal, though the germ cells are slightly degenerated and the ovarian cavity is a little narrowed. In one of them oocytes somewhat
enlarged were found. The gonad expressed by Fig. 22 is very interesting; many germ cells are grouped in the distal half and some are in the state of elimination. The distal part of this gonad is bifurcated, each branch mainly consisted of germ cells. In the proximal portion the anlage of sex-cord is observable. The gonad seems to be in the process of sex-reversal from female to male.

The sixth group of the experimentals includes 7 individuals, 48–57 mm. The larvae belonging to this group were all (except one) metamorphosed. Out of 7 specimens 5 are males; their testes are mostly normal and well-developed, but two of them have hyaline part. One of these gonads with the hyaline part has germ cells which are not so active and are arranged somewhat in disorder. There are two females in this group: the ovary of a specimen is somewhat degenerated, their germ cells being inactive and partially eliminated. The ovarian cavity is a trifle diminished. The large oocytes are scarce in number and some are eliminated. The another ovary has active germ cells and rete cells, and no more elimination is observable. The ovarian cavity, however, is entirely packed with large oocytes and many rete cells, showing approach to the mosaic gonad (Fig. 23). The immigration of the rete cord is remarkable but no sex-cord is found.

The result of the present experiment has been compared with that of the homoiotransplantation of H. lichenatus as in the following:

1. In the early stage (25–35 mm) the medullary immigration of germ cells is remarkable in homoiotransplantation. In the heterotransplantation the medullary immigration is not pronounced and the modified free-martin gonads, though rare, are met with.

2. In later stages the difference is more distinct. In the homoiotransplantation the male gonads become activated to develop. The female gonads are provided with medullary rete cells or with the cortical germ cells degenerated. In the heterotransplantation the male gonads are generally small in size and with scarce germ cells as seen in the normal development. The female gonads mostly take the shape of the modified free-martin condition, with active germ cells aggregated in the distal portion or of the condition of affected ovary, with the core of rete cells in the medulla.

3. In the larvae of H. lichenatus just before metamorphosis and ingrafted with the testicular piece of the same species, the male gonads are all activated to rapid growth, while the female gonads have always the ovarian cavity packed with medullary rete cells and large ovicells which are often situated in the medulla. In the lichenatus-larvae of the similar stage, ingrafted with the testicular piece of H. retardatus, the male gonads are rather small and have hyaline parts containing cells sparsely distributed, while the female gonads are partly normal, though
the ovarian cavity is often narrowed, and partly affected, with the medulla solid with rete cells. The medullary immigration of rete cells is not so remarkable in the heterotransplantation as in the homoiotransplantation, because in the former case the cortical germ layer is very thick as in the normal female gonad because of slight elimination or degeneration of germ cells. In these female gonads large ovicells are rather rarely found, as the activation is not so violent as in the homoiotransplantation.

The results of the two experiments lead to the conclusion that the testicular graft of *H. lichenatus* is more effective than that of *H. retardatus* to the elimination and degeneration of germ cells in the female gonads.

**Discussion**

The sex-reversal of *Hynobius* gonads from female to male will be analysed into the following two morphological phases: cortical degeneration and medullary development. The cortical degeneration is a phenomenon due to the elimination or degeneration of germ cells and rete cells. The medullary development is dependent upon the ingrowth of rete cells and sex-cords. When following the cortical degeneration, the medullary development occurs compensatively, the sex-reversal is completed.

So far as the writer observed, the elimination and degeneration of germ cells do not always take place synchronously. They may be caused by two different factors. Ingrowth of rete cells and germ cells generally occurs incomitant with the cortical degeneration and especially as a compensatory phase after the cortical degeneration. But there are some female gonads of which cortical cells are nearly degenerated but no immigrant rete cell is found. Moreover, some cortical germ cells seem to be shifted toward medullary portion. Therefore, the medullary immigration seems to depend on another factor. These three factors seem to play the important rôles on the sex-reversal from female to male. Besides these factors, there is a remarkable fact that the germ cells are often activated by testicular graft or by injection of follicular hormone to large ovicells in the relatively young stage. This seems to be another factor sometimes effective in the sex-reversal from male to female. The mosaic gonads are produced both from the male and the female gonads. When the follicular hormone is injected to the male, some of the germ cells are activated to become large ovicells, thence, they form together with the sex-cords the mosaic gonads. On the other hand, when the ovaries are affected by the strong male hormone, many germ cells were eliminated and some became activated to large ovicells and then sex-cords were formed in the rete cells invaded to the medullary portion, hence the formation of the mosaic gonads of female nature. At any rate, the mosaic
gonads are formed, when the balance of these effects may be suitably retained.

In the both species, *Hynobius retardatus* and *H. lichenatus*, the homoiotransplantations of the testicular grafts on their larvae are on the whole similar in results. In these experiments the male gonads were always accelerated to develop, while the female gonads received the following effects; elimination of cortical germ cells, medullary immigration of rete cells (sometimes with germ cells), and activation of female germ cells to rapidly grow to large ovicells. But when the reciprocal testicular graftings were made, the gonads of the both recipients do not follow the category above mentioned. For instance, in the female gonads of *H. retardatus* with the testicular graft of *H. lichenatus* the medullary immigration of rete cells does not occur always, but the elimination and degeneration of cortical cells are violent, thus forming the ovary with degenerated cortex but with the ovarian cavity. On the other hand, in the female gonads of *H. lichenatus* with the testicular graft of *H. retardatus* the elimination and degeneration of cortical cells are very faint, so the thick cortical layer seems to interfere the rapid medullary ingrowth of rete cells. Some of these female gonads ingrained with *retardatus*-testicular pieces were slightly affected, with the normal cortex and a large ovarian cavity.

Judging from the results just referred, the effects of testicular grafts from the different species on the gonads of the hosts are different. The difference of the effect seems to be proportional to both the intensity and amount of hormone which exerts. In the present experiment the amount of the testicular grafts is approximately similar, so the diverse results are attributable to the intensity of the hormone produced from the grafted testes. The hormone of the differentiated species (*H. lichenatus*) seems to be more effective than the hormone of the semi-differentiated species (*H. retardatus*). The gonads of hosts are always more influenced by the testicular grafts of *H. lichenatus* than that of *H. retardatus*. The results seem to be quite coincided with the results of the parabiosis experiment between the two salamanders (1941) above mentioned. The testicular grafts of *H. lichenatus* cause the elimination, degeneration and activation of female germ cells of the gonads of the host species, *H. retardatus*. The elimination of germ cells effected by the testicular grafts is not only seen in the female but also in the male gonads, and is almost observable through the whole stage from the beginning to the differentiation of the gonads. The elimination seems to become very progressive by excess of hormone amount. The experiments of testosterone-propionate injection upon several species of Amphibians are very diverse in results (Gallien, 1937; Witschi & Crown, 1938; Burns, 1938, 1939; Puckett, 1939, 1940; Foot, 1940; Hanaoka, 1941). It seems to be
probable that the effective amount of the hormone is different according to amphibian species. The sterile gonads produced by the hormone injection in Hanaoka's experiment (1941) are obviously due to the injection of excess hormone. The degenerated female gonads in this experiment are often represented by a scanty layer of inactive germ cells surrounding a large ovarian cavity. The female gonads can be deduced from the degenerated ovaries of the hermaphrodite species, *H. retardatus* ingrafted with the testicular graft of the gonochoonistic species, *H. lichenatus* (Fig. 8). It is also very peculiar that the ovaries of *H. retardatus* affected by the testicular grafts of *H. lichenatus* are mostly characterized in the degeneration of cortical elements, though the ovarian cavity is wide as in Fig. 7. The degeneration of the germ cells is also due to the effect of excess hormone from the graft of *H. lichenatus*. In Hanaoka's experiment the male gonads also became sterile possibly on account of violent elimination. If the amount of the hormone is moderate, the elimination might not be so frequent and the male gonads will be normally accelerated to develop. Besides Hanaoka's experiment, Witschi (1936) who united the embryos of *Amblystoma tigrinum* with those of differentiated (gonochoonistic) race or with those of undifferentiated (hermaphrodite) race of *A. maculatum* pointed out as follows: In the heterosexual combination (*tigrinum ♂ × maculatum ♀*) the suppression of ovarial differentiation was very complete in two races of *maculatum*. But in the homosexual combination (*tigrinum ♂ × maculatum ♂*), the results are entirely different in two races. If the *maculatum* member belongs to the differentiated race, the testes of the two species develop like those of simple controls, whereas the testes of the semidifferentiated *maculatum* twin were very small, of atypical structure and nearly (or in extreme cases completely) sterile. In our parabiosis experiment (*H. retardatus × H. lichenatus*) the male-male combinations mostly developed normally, but the elimination of germ cells is very frequently observed in the testes of *H. retardatus* alone.

**Literature**

3. — 1941. The effect of testosterone-propionate upon the sex differentiation in *Hynobius retardatus*. *Ditto*, vol. 7, no. 4, pp. 413-419, pls. 33-34.

