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Separation of two species standing as *Helophorus aquaticus* (L.) (Coleoptera, Hydrophilidae) by banded chromosome analysis

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ABSTRACT. Methods are described for making chromosome preparations from developing embryos of *Helophorus,* for producing C- and G-banding, and for staining the nucleolus organizer with silver. These methods are used to compare the karyotypes of two species currently included in *H.aquaticus* (L.). It is shown that these species differ because of reciprocal translocations between some chromosomes, and that they would therefore be unable to produce fertile hybrids. Morphological differences in the male and female genitalia are described, and the range of aedeagal variation shown by each species is established by reference to chromosome preparations from testis. Reference to the relevant type specimens shows that the two species are *H.aequalis* Thomson and *H.aquaticus* (L.). The latter is not a British species. Differences in the egg cocoons and third instar larvae are described. The present distributions and Pleistocene histories of the two species are described.

Introduction

Helophorus aquaticus (L.) as currently interpreted (Angus, 1970), is a common species of water-beetle, widely distributed in Europe as far east as the Urals. It is typically found in small muddy pools in winter and spring, and feeds on filamentous algae as well as grass and other vegetation which has begun to decay following inundation. The life history and immature stages are described by Angus (1973b). The beetle is of interest in that not only is it a common species in Britain today, but it is of frequent occurrence in Pleistocene faunas studied by G. R. Coope and his colleagues at Birmingham University.

The existence of two apparent races of this species, differing in the shape of their parameres, was reported by Angus (1973a). The two forms have been regarded as subspecies because in those areas of Europe where their ranges coincide they appear to show morphological intergradation. However, study of the chromosomes shows that the two are in fact good species, and that all the aedeagal variation is produced by these two species without hybridization. Study of the types indicates that the British species, which has relatively longer parameres with straight outer margins, is *H.aequalis* Thomson. The other species, with shorter parameres with curved outer margins, is *H.aquaticus* (L.) and does not occur in Britain.

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The karyotypes

Material

Good quality preparations of mitotic chromosomes are most readily obtained from developing embryos. The biology of the beetles facilitates this approach as the eggs are laid in batches of about twelve to sixteen in silk cocoons placed in the mud at the edges of the pools where the beetles live (Angus, 1973b). In England *H.aequalis* normally starts to lay eggs in November or December, depending on when the autumn rains fill the small pools which have dried out

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during the summer. Breeding continues until the weather turns hot and the pools dry out, usually in May. Breeding ceases during frosty periods, and, in years with a severe winter, is confined to the spring. French material was collected in late March, when breeding was found to be in progress.

The material used for chromosome preparations is from the following localities.

ENGLAND. Surrey: Runnymede; Middlesex: Staines Moor (*H.aequalis*).
FRANCE. Indre et Loire: Neuillé-Pont-Pierre, Sonzay, Sorigny (both species), Souvigné, Tournon St Martin (*aequalis*); Cher: Culan (both species); Indre: Scoury and the adjacent Brenne region (*aequalis*); Puy de Dôme: Pontaumur, Pouzol, St Pardoux (both species); Creuse: Fontanières (both species); Vienne: St Savin (both species); Cantal: St Flour (*aequalis*); Aude: Castelnaudary (*aquaticus*).
SPAIN. Provincia de Segovia: La Granja, Valsain (*aquaticus*).

Methods

Egg cocoons are either collected in the field or obtained from beetles kept in aquaria in the laboratory. At temperatures of about 18°C the eggs hatch a week after being laid. The optimum stage for chromosome work is reached after 2–3 days. Most of the eggs are then removed from the cocoon for processing, but some are left and reared through to check their identity.

Two methods have been used to prepare chromosome spreads.

1. *Acetic acid spreading.* This is a modified version of the method described by Crozier (1968). Batches of eggs are placed in solid watch glasses of 0.1% colchicine in 0.75% NaCl, buffered to pH 6.8 with Sörensen's phosphate buffer (M/150). The eggs are pierced with a sliver of glass and left for 15 min in darkness. They are then removed with a Pasteur pipette and transferred to a second watch glass containing 0.05% trypsin in 0.48% KCl, again buffered to pH 6.8. The eggs are squeezed between fine forceps, so that the contents are extruded through the holes in their shells. They are then returned to darkness and left for 30 min, after which they are pipetted into watch glasses of freshly prepared 3:1 absolute ethanol:acetic acid. The fixative is changed twice and the embryos are fixed for 30 min. Pieces of tissue are then transferred to clean slides with fine forceps. A small drop of 45% acetic acid is added from a hypodermic needle, before the tissue dries. The drop of acid, with its cell suspension, is examined under the microscope to check the dissociation of the cells. If necessary, it can be agitated with a fine needle. One drop of fixative is then dropped on to the suspension, and the mixture is then allowed to spread across the slide before drying.

2. *Spreading directly from fixative, following centrifugation.* In this method the treatments with colchicine and trypsin in hypotonic KCl are carried out as above. The inflated embryos are then transferred, in as little saline as possible (about 0.1 ml), to a 5 ml centrifuge tube. Ice cold 4:1 absolute methanol:acetic acid fixative is then added, the pipette being inserted right to the bottom of the tube so that the mixture is thoroughly stirred by the introduction of the fixative. This prevents the cells from sticking together. The tube is filled with fixative (about 4 ml). It is important that the saline does not dilute the fixative by more than about 2.5%, otherwise the cytoplasm stains heavily. The cells are left in the fixative for 30 min, then centrifuged at 120 *g* for 10 min. The supernatant is pipetted off, the pellet of cells is agitated, fresh fixative is added, and the tube is centrifuged again. One further change of fixative is used, then, after the final centrifugation, the pellet of cells is suspended in about 0.1 ml of fixative at the bottom of the tube. This is pipetted on to dry slides which have been cleaned in dichromic acid and rinsed in distilled water. The slides are tilted to control the spread of the cell suspension before it dries.

Banding

C-banding. The most reliable method of producing C-banding involves immersing 3–7-day-old slides, prepared by acetic acid spreading, in a saturated solution of $Ba(OH)_2$ for about 5 min at room temperature. The slides are then rinsed three times in distilled water buffered to pH 6.8 with Sörensen. They are then incubated for 1 h in 2 × SSC (0.3 M sodium chloride and 0.03 M trisodium citrate),

before staining in 2% Giemsa in distilled water buffered to pH 6.8.

G-banding. Although G-banding is a standard technique in the analysis of mammalian chromosomes, there are few instances of its use with insects. One reason for this is that the methods used for producing G-banding in mammalian chromosomes have to be drastically modified to work with insects. Although I have found that a number of treatments will produce what appears to be G-banding in *Helophorus* chromosomes, the most useful appears to be a trypsin treatment, as follows. Freshly prepared slides, made with the acetic acid spreading method described above, are allowed to dry for about 5 min, then immersed in a 0.01% solution of trypsin (Difco, 1 : 250) in 0.75% NaCl buffered to pH 7.6 with Sörensen, for 5–15 s at 10°C. They are then rinsed three times in distilled water buffered to pH 6.0, after which they are stained for about 20 min in 2% Giemsa at pH 6.8. The pH values are critical for controlling this very slight trypsin treatment. Older slides cannot be G-banded by this schedule. A range of treatment times is necessary, and where the treatment has been successful many nuclei will show the G-banding, but only a few will be of definitive quality. The alkaline saline solution will band the chromosomes without trypsin, but this requires 30 s to 1 min, at temperatures of about 25°C.

In view of the difficulties encountered in obtaining G-banding, and also because of the rather regular nature of the pattern produced, it was considered necessary to test the reality of the banding patterns by using the antibiotic treatments of living cells described by Rønne and his colleagues (Rønne, 1977; Rønne & Andersen, 1978). Best results were obtained with 5-fluorouridine, which was obtained from Calbiochem. The treatment was as follows. Eggs were placed in a watch glass of 0.75% NaCl, buffered to pH 6.8 with Sörensen, containing 15–20 μg 5-fluorouridine per ml. The eggs were pierced with a glass sliver, and additional 5-fluorouridine solution was injected into the eggs using a glass microelectrode fastened to a hypodermic syringe. The eggs were then left in darkness for 4 h, after which they were transferred to colchicine and chromosome preparations made using the acetic acid spreading technique already described. The slides were allowed to dry for 15 min, then stained in 2% Giemsa at pH 6.8.

The effect of the treatment on the chromosomes was uneven. In some cells the chromosomes were unaffected, and in others the chromosomes were grossly overcontracted. However, in a number of cases very clear G-banding patterns, similar to those produced by postfixation treatments, were produced. In other cases an 'uncoiling' effect, similar to that described by Rønne & Andersen (1978), was observed. Thus treatment with 5-fluorouridine not only confirms that the G-banding is a real phenomenon reflecting the structure of the chromosomes, but it can also produce very clear and detailed G-banding, enabling critical comparisons of individual chromosomes to be made (Fig. 4e and f). The effects of the various banding treatments are shown in Fig. 3, using *H.aequalis,* chromosome 7. The relationship between 'uncoiling' and G-banding is discussed by Takayama (1976). The 'uncoiling' appearance can be produced by postfixational treatments, and it may be seen that while the karyotype of *H.aequalis* shown in Fig. 1 shows well-developed G-banding, that of *H.aquaticus* shown in Fig. 2 shows a condition to some extent intermediate between banding and 'uncoiling'. If the model of chromosome structure put forward by Bahr (1977), as a result of electron microscope studies, is correct the apparent helical structure suggested by 'uncoiling' must be spurious.

It will be seen from Fig. 3, a and b, that while control preparations made by acetic acid spreading show, at most, traces of G-banding, this banding is more apparent when freshly made preparations done by the centrifugation method are stained with Giemsa. This method of obtaining the banding was used by Steiniger & Mukherjee (1975) with mosquito chromosomes. Here the banding was intensified by reducing the fixation time, but this has the disadvantage of producing a more irregular banding.

Staining the nucleolus organizer

The nucleolus organizer of *H.aequalis* has been silver-stained using the method of Goodpasture & Bloom (1975) on 1- or 2-day-old slides prepared by the centrifugation method,

using 3:1 ethanol:acetic acid as the fixative. The nucleolus organizer is associated with the secondary constriction of chromosome 6, while resting nuclei show two nucleoli (Fig. 18). This method has only occasionally been successful, but when it does work the results are consistent. I have not stained the nucleolus organizer of *H.aquaticus*, but since *H.grandis* Ill. also shows the nucleolus organizer to be associated with the single prominent secondary constriction, it is concluded that the secondary constriction of the *H.aquaticus* chromosome 6 is also associated with the nucleolus organizer.

Mounting, examination and photography

After staining, the slides were rinsed in distilled water, excess water was shaken off, and the slides were placed vertically in racks over a hot plate. Once all visible water had evaporated, they were transferred to a desiccator at room temperature and left for at least 12 h. The preparations were mounted in Loctite 358 polymerizing resin, which was set by exposure to ultraviolet light. The resin is applied to the coverslip, and this is then pressed on to the preparation, causing the resin to spread to the edges. In this way the resin can be set hard within a minute of reaching the chromosomes, which eliminates the leaching of the stain by the liquid mounting medium. This is the only method which has been found to be satisfactory with trypsin-induced G-banding. Some preparations are as good as new after 2 years. This method is not suitable for phase-contrast work.

The slides were examined with a Leitz Orthoplan photomicroscope, using a Leitz Fluortar 100/1.32 oil immersion lens, and a Zeiss precision interference filter No. 467808 to give a monochromatic green light. Partial closure of the condenser helps to emphasize the banding. Preparations were photographed on to Ilford Ilfodata HS 23 Type J 500 film, with the exposure meter set to 10 or 11 DIN (8–10 ASA). The film was developed for 5 min in 1 part Ilfodata CP developer in 5 parts water at 20°C. This gives a good contrast range, and avoids graininess.

Measurement

Measurements were made from photographs printed at ×4800. A micrometer was made by fastening a cog wheel to the winder of an old wrist watch, and the minutes of the clock face were taken as units of measurement. Running the cog wheel along the chromosomes enables curved chromosomes to be measured accurately. For each chromosome the relative chromosome length (the length of the chromosome divided by the total haploid autosome length and multiplied by 100) and the centromere index (the length of the short arm divided by the length of the chromosome and multiplied by 100) were calculated.

Results

In both species the diploid chromosome complement is 18, 16 autosomes + XY (♂), XX (♀). The Y-chromosome is a small metacentric, appearing as a dot in many preparations. The relative chromosome lengths and centromere indices of the chromosomes are given in Table 1. C-banded preparations are shown in Figs. 16 and 17, G-banded karyotypes in Figs. 1 and 2, and the silver stained nucleolus organizers of *H.aequalis* are shown in Fig. 18. Diagrams of the chromosomes, showing all these features, are shown in Figs. 19 and 20.

H.aequalis. No variation has been found in the karyotype of this species. The chromosomes are numbered according to size, except that chromosome 6, although slightly shorter than 7, is placed before it to facilitate comparison with other species. Most of the chromosomes are fairly distinctive because of their sizes and centromere positions, but chromosomes 3 and 4 present difficulties, both in distinguishing one from the other, and also in recognizing the long and short arms. The G-banding patterns are, however, characteristic. Chromosome 4 is most easily recognized by the pair of rather heavy bands, separated by a narrow gap, in the middle of the long arm. Chromosome 3 has a band in the middle of its long arm (Fig. 1).

H.aquaticus. This is a more difficult karyotype to interpret, partly because the small and medium-sized chromosomes are less distinctive in size and centromere position, and partly because of the polymorphism shown by chromosome 7. As in *H.aequalis*, the chromosomes are numbered according to size, except

TABLE 1. *Helophorus aequalis* and *aquaticus*, chromosome dimensions.

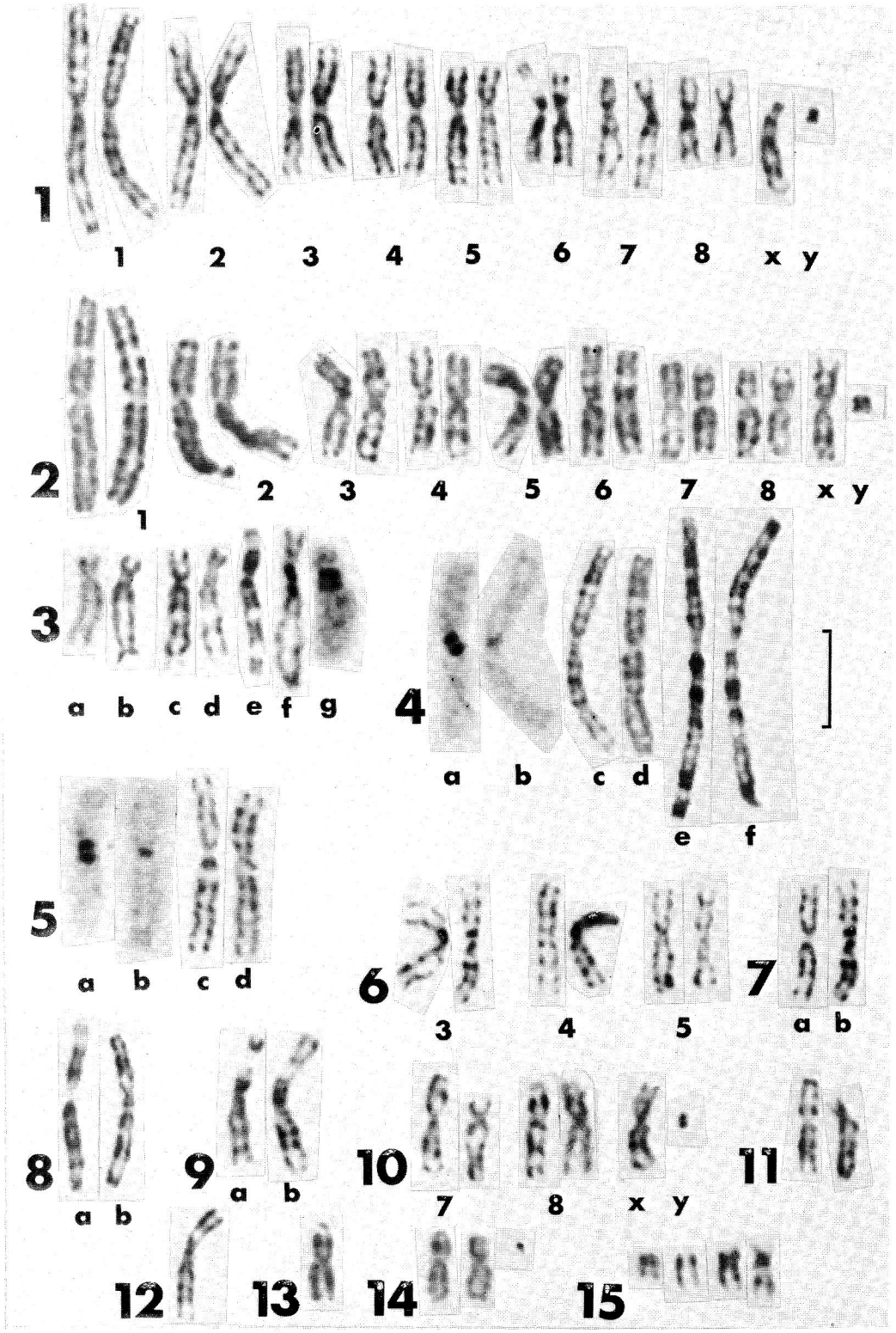
Chromosome	Relative chromosome length and 95% confidence limits	Centromere index and 95% confidence limits
H.aequalis		
1	20.88 (20.47–21.29) $N = 30$	45.48 (44.44–46.52) $N = 25$
2	17.31 (16.97–17.66) $N = 30$	41.67 (40.64–42.69) $N = 24$
3	12.10 (11.86–12.34) $N = 29$	44.78 (43.55–46.01) $N = 27$
4	11.38 (11.18–11.57) $N = 29$	43.76 (42.62–44.90) $N = 25$
5	11.15 (10.88–11.42) $N = 29$	34.82 (33.34–36.30) $N = 28$
6	9.44 (9.01–9.86) $N = 28$	44.82 (42.48–47.16) $N = 28$
7	9.52 (9.22–9.82) $N = 28$	21.25 (18.96–23.54) $N = 28$
8	8.19 (8.00–8.37) $N = 28$	38.14 (36.74–39.55) $N = 28$
X	8.34 (7.97–8.70) $N = 20$	13.35 (12.25–14.45) $N = 20$
H.aquaticus		
1	20.41 (19.99–20.83) $N = 32$	44.54 (43.62–45.45) $N = 28$
2	16.81 (16.52–17.10) $N = 33$	38.08 (37.28–38.88) $N = 25$
3	11.43 (11.20–11.66) $N = 35$	45.56 (44.74–46.38) $N = 32$
4	10.57 (10.37–10.76) $N = 35$	46.34 (45.45–47.24) $N = 35$
5	10.63 (10.38–10.89) $N = 34$	41.15 (39.80–42.50) $N = 33$
6	11.36 (11.10–11.63) $N = 34$	47.53 (46.44–48.63) $N = 30$
7 metacentric	9.53 (9.27–9.79) $N = 32$	42.31 (40.96–43.66) $N = 29$
7 acrocentric	8.83 (8.35–9.30) $N = 4$	21.75 (16.34–27.16) $N = 4$
8	8.99 (8.80–9.19) $N = 36$	37.94 (36.78–39.10) $N = 34$
X	9.23 (8.92–9.53) $N = 23$	44.48 (43.22–45.74) $N = 21$
B	3.70 (0.22–7.18) $N = 3$	–

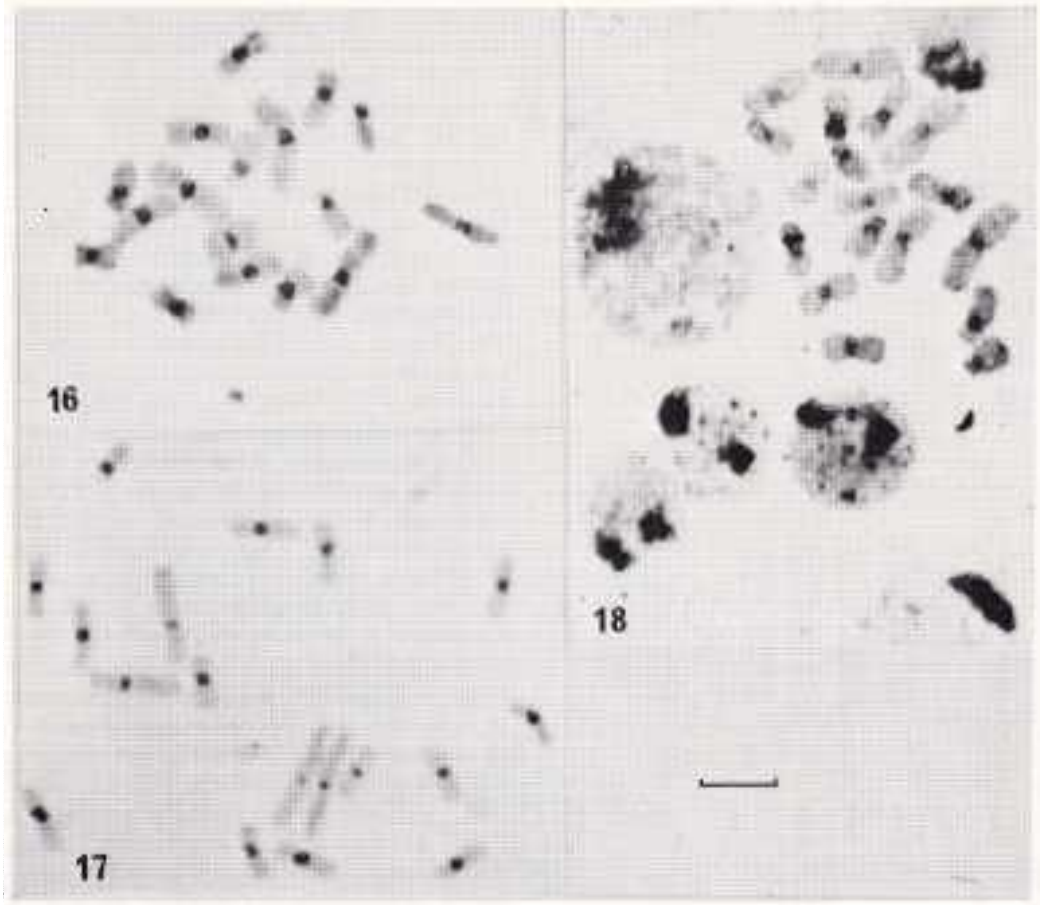
that chromosomes 5 and 6 are numbered to facilitate comparison with *aequalis*. In addition to the G-banded karyotype shown in Fig. 2, detailed comparisons of chromosomes 3, 4 and 5 are shown in Fig. 6, and of chromosomes 7, 8 and X, with the polymorphism of 7, in Fig. 10. The relationship between metacentric and acrocentric chromosome 7 is further illustrated in Fig. 11. Although the material at present available does not give a definitive explanation of the transformation of one form to the other, the position of the double band in the long arm of both acrocentric and metacentric forms suggests that, rather than a pericentric inversion, there has been a translocation of material from the distal part of the short arm to the long arm. It will be noted from Table 1 that acrocentric 7 appears slightly shorter than the metacentric, and, despite the small sample, this is supported by additional observations, particularly of more contracted chromosomes. Fig. 10 shows that the acrocentric is not chromosome 8, while its separation from the X-chromosome is confirmed by the meiotic second metaphase shown in Fig. 28, where the acrocentric is shown in the same haploid nucleus as the Y-chromosome.

The *H.aquaticus* karyotype also varies in the presence of one or two B-chromosomes (Figs. 15, 25 and 28) in some individuals. Beetles with B-chromosomes may have either no B-chromosomes, or one, or two, in different nuclei. In addition to the B-chromosomes, some specimens have a small satellite associated with chromosome 8 (Fig. 13). In only one preparation has a satellite been found unattached (Fig. 14). The acrocentric form of chromosome 7, the B-chromosomes and the satellite have been found in most of the French localities, but not in Spain. No specimen has been found to be homozygous for acrocentric 7.

Comparison of the karyotypes of H.aequalis and aquaticus

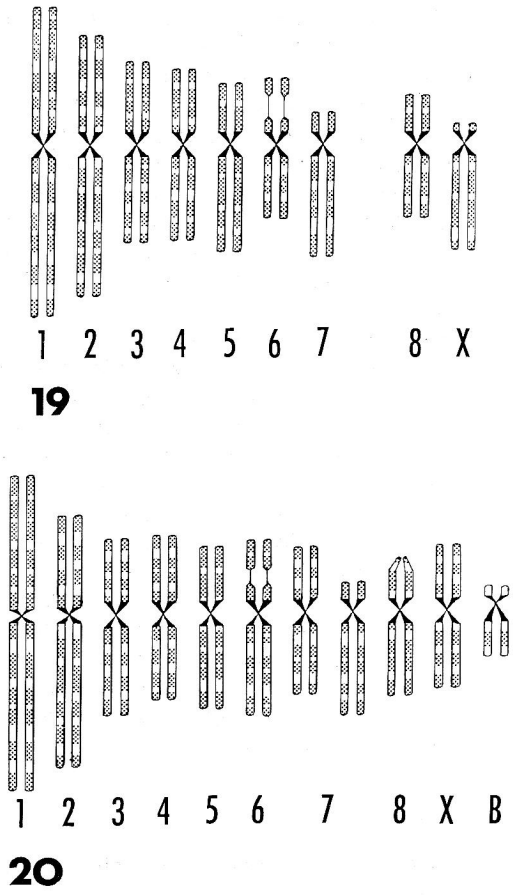
It will be seen from Table 1 that there is very little agreement between the relative chromosome lengths and centromere indices of the chromosomes of the two species. Only


1
1 2 3 4 5 6 7 8 x y
2
1 2 3 4 5 6 7 8 x y
3
a b c d e f g
4
a b c d e f
5
a b c d
6
3 4 5
7
a b
8
a b
9
a b
10
7 8 x y
11
12
13
14
15



FIGS. 16–18. *H.aquaticus* and *aequalis* chromosomes. The scale bar represents 5 μm. 16, *H.aequalis* from Sorigny, C-banded with $Ba(OH)_2$. 17, *H.aquaticus* from St Pardoux, C-banded with NaOH. 18, *H.aequalis* from St Savin, silver stained to show the nucleolus organizers on chromosome 6, and the C-bands on all chromosomes. Note the paired nucleoli in the resting nuclei.

FIGS. 1–15. Chromosomes of *Helophorus aequalis* and *aquaticus*. The scale bar represents 5 μm. 1, *H.aequalis* from St Savin, karyotype G-banded with trypsin. 2, *H.aquaticus* from Fontanières G-banded with trypsin. 3a–g, *H.aequalis*, chromosome 7, various treatments: a, control spread with acetic acid; b, control spread directly from fixative, following centrifugation. c, G-banded with trypsin; d, G-banded with alkaline saline; e, G-banded and, f, uncoiled, following treatment with 5-fluorouridine; g, C-banded with NaOH. 4a–f, *H.aequalis* and *aquaticus*, chromosome 1: a, *aequalis* and b, *aquaticus*, C-banded; c, *aequalis* and d, *aquaticus*, G-banded with trypsin; e, *aequalis* and f, *aquaticus*, G-banded with 5-fluorouridine. 5a–d, *H.aequalis* and *aquaticus*, chromosome 2: a, *aequalis* and b, *aquaticus*, C-banded; c, *aequalis* and d, *aquaticus* G-banded. 6, *H.aquaticus*, chromosomes 3, 4 and 5, G-banded. 7a–b, G-banded chromosomes: a, *H.aequalis*, chromosome 4; b, *H.aquaticus*, chromosome 3. 8a–b, Chromosome 5, G-banded with 5-fluorouridine, from the same nuclei as Fig. 4, e and f: a, *H.aequalis*; b, *H.aquaticus*. 9a–b, Chromosome 6, G-banded and similarly extended: a, *H.aequalis*; b, *H.aquaticus*. 10, *H.aquaticus*, G-banded chromosomes 7, 8, X and Y. 11, *H.aquaticus*, metacentric and acrocentric chromosome 7 from different nuclei, G-banded and contracted to the same degree as those shown in Fig. 10. 12, *H.aequalis*, chromosome 8 G-banded with 5-fluorouridine. 13, *H.aquaticus*, chromosome 8 with attached satellite. 14, *H.aquaticus*, G-banded chromosome 8 with attached and unattached satellites. 15, *H.aquaticus*, B-chromosomes with differing degrees of contraction. G-banded.



FIGS. 19 and 20. Diagrams of the banded chromosomes of *H.aequalis* (19) and *H.aquaticus* (20). C-bands are shown black, G-bands stippled, the secondary constriction as a single line and the satellite association of *H.aquaticus* chromosome 8 by convergence of the outer ends of the short arms.

in the case of chromosome 1 do the figures appear similar, and Student's *t*-test shows no difference between them at the 95% level of significance. If chromosome 1 is homologus, and therefore the same actual length, in the two species, as well as having the same relative chromosome length, then there must be the same amount of autosomal material in the two species. The differences in relative lengths between the other chromosomes of the two species would have to be the result of translocations of material between chromosomes, which would prevent normal pairing at meiosis in any hybrids, resulting in their infertility. The extent of the homology may be checked by reference to the banding.

When comparing G-banding it must be remembered that the pattern of bands is affected by the degree of contraction of the chromosomes, which is not always the same along the length of a chromosome. The banding also varies from a condition approaching uncoiling to one in which smaller bands may be lost as a result of overtreatment with the banding agent. This means that many preparations are not suitable for critical comparison, but where a number of clear preparations are available, some similarities and differences between chromosomes may be detected.

C-banding shows that chromosome 1 of *aequalis* has about twice as much heterochromatin at the centromere as that of *aquaticus* (Figs. 4, a and b, 16 and 17). This should give *aequalis* a larger chromosome. However, in G-banded preparations (Figs. 1, 2 and 4, c–f) the short arm is seen to have a narrow gap which is about half way along its length in *aequalis*, but slightly nearer the base in *aquaticus*. Fig. 4(e) shows chromosome 1 of an *aequalis* from Staines Moor, banded by 5-fluorouridine, in which the banding is very clear. Fig. 4(f) shows a similar preparation from an *aquaticus* from Valsain. These preparations show the smaller centromere region of *aquaticus*, and also that the part of the short arm basal to the median gap, as well as the long arm, at least in its basal half, are homologous. The distal half of the long arm in Fig. 4(f) is not clear, but if Figs. 4(c–f) are all considered, it seems likely that the whole of the long arm has the same banding pattern, and is therefore homologous in the two species. However, the apical section of the short arm, distal to the median gap, is clearly not homologous. The three even bands in *aquaticus* do not match the two, often subdivided, bands in *aequalis*. It must therefore be concluded that this section of the chromosome has been involved in a translocation. However, the increase in length of the long arm of *aquaticus* compared with *aequalis* appears to be similar to the decrease in chromosome length caused by the smaller heterochromatic C-banding region. Thus it seems that chromosome 1 is the same size in the two species, and that there must therefore have been extensive translocation to account

for the different relative chromosome lengths shown in the two karyotypes.

Figs. 8(a) and 8(b) show chromosome 5 of *aequalis* and *aquaticus*, from the same nuclei as the chromosomes shown in Figs. 4(e) and 4(f). The short arm is the same size in the two species, and the banding appears homologous. The long arms, however, are quite different, that of *aequalis* having an arrangement remarkably like the three even bands in the apical portion of the short arm of chromosome 1 of *aquaticus*, while the two bands in the long arm of the *aquaticus* chromosome 5 bear a similar resemblance to those in the apical portion of the short arm of the *aequalis* chromosome 1. Although it cannot be taken as certain that there has been a reciprocal translocation between chromosomes 1 and 5 of the two species, it does seem likely, the more so as not only do the short arms of chromosome 5 appear homologous in the two species, but no other chromosome arm in either species matches this short arm.

Comparison of chromosome 2 in the two species shows that in *aquaticus* the chromosome appears shorter, and with a relatively shorter short arm. C-banding shows that in *aquaticus* the heterochromatic centromere region is considerably smaller than in *aequalis* (Figs. 16 and 17, and Fig. 5, a and b). G-banding (Fig. 5, c and d) shows that the long arm is homologous in the two species, but that at first sight the short arm of *aequalis* has one more band than in *aquaticus*. Reference to the C-banding, however, shows that while the end of the centromere is what it appears to be in the *aequalis* preparation, in *aquaticus* it is the basal G-band which is closely applied to to the smaller centromere region. This also accounts for the obvious similarity between the bands in the middle of the short arm in the two species. It is not clear why the reduction in the heterochromatic centromere region of *aquaticus* should result in a relatively shorter short arm. The C-banded preparations do not really suggest that heterochromatin is concentrated in the long arm of *aquaticus*, though they do not rule this out. The same consideration applies to chromosome 1 of the two species, where the centromere index appears the same in the two species, despite the longer apical section of the short arm in *aquaticus*.

The only other pair of chromosomes which may be homologous in the two species are chromosome 4 of *aequalis* and chromosome 3 of *aquaticus*. The relative chromosome lengths show no significant differences, but the centromere indices, though similar, give the *aquaticus* chromosome 4 a significantly longer short arm. G-banded preparations of these two chromosomes are shown in Fig. 7, and it will be seen that the banding pattern appears the same in the two.

The remaining chromosomes do not appear homologous in the two species. Fig. 9 shows similarly expanded chromosome 6 from the two species. The secondary constrictions are presumably homologous, but the rest of the chromosomes show that, despite the regular pattern of the G-banding, the arrangements do not match in the two species. The short chromosomes, 7 and 8, do not have sufficiently extensive banding patterns for partial homologies to be investigated. The X-chromosome of *aequalis* is distinctly shorter than that of *aquaticus*, but again the banding pattern does not indicate the extent of any homology between the two species.

Discussion

Comparison of the relative chromosome lengths of the two species, in conjunction with the banding patterns of the chromosomes, shows that the two karyotypes differ by a number of translocations, and that they must therefore be regarded as separate species. The banding patterns reveal one case (chromosome 2) where a difference in size and centromere index results merely from a different amount of juxta centromeric heterochromatin, the euchromatic parts of the chromosomes being homologous, and one case (chromosome 1) in which, despite similar relative chromosome lengths and centromere indices, the chromosomes are only partly homologous. The G-banding is also useful in enabling certain chromosomes to be identified, and in investigating the polymorphism of chromosome 7 of *H.aquaticus*. The variation shown in the karyotype of *H.aquaticus* illustrates the need to demonstrate the existence of interchromosomal translocation differences between the karyotypes of the two species before their status as separate species can be validated.

The variability of the species

As mentioned in the Introduction, the two species show an apparent morphological intergradation where their ranges overlap. In view of the chromosomal separation of the two as distinct species it is therefore necessary to establish the extent of variation attributable to each species, and to ascertain whether the intergradation is the result of natural hybridization. Some information on the variation within each species can be obtained from study of specimens from areas where only one species is present, and from specimens reared from a single cocoon. Figs. 38 and 39 show the aedeagophores of two *aequalis* from the same cocoon laid by a female from Souvigné, and while it may be seen that the parameres shown in Fig. 38 are of normal *aequalis* pattern, those in Fig. 39 are broader and with their outer margins slightly curved.

The most useful method of analysing the variation is to examine the chromosomes in the testes of young males. The testes show active mitosis and meiosis when the beetles have been emerged for about 2–3 months. After this the testes contain only sperm. In central France both species emerge at the beginning of June, and specimens collected at this time (from localities already listed) were brought back and kept in aquaria. Chromosome preparations were made using the acetic acid spreading method, with the beetles injected with colchicine about 20 min before dissection. The abdomens, with the aedeagophores, were placed for 1 h in 70% alcohol to harden the aedeagophores to avoid distortion when they were mounted on slides. Such distortion often occurs if fresh material is mounted directly into D.M.H.F., following the method described by Angus (1970). The aedeagophores, once hardened, were dehydrated and mounted in balsam. This avoided all distortion of paramere shape.

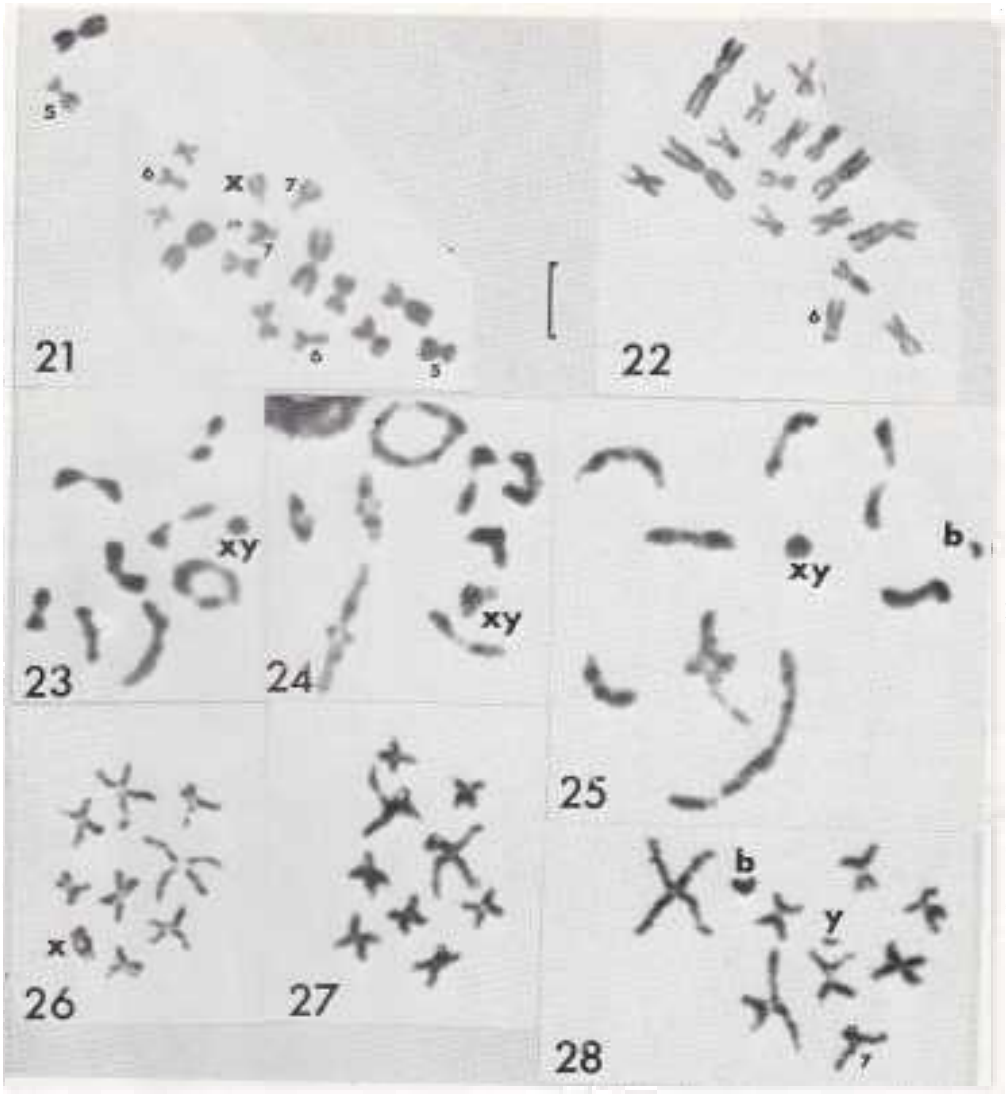
Chromosome preparations from testes are shown in Figs. 21–28. Although G-banding proved virtually impossible to produce, the chromosomes in both spermatogonial mitosis and in metaphase of the second meiotic division are distinctive. The acrocentric X-chromosome of *aequalis* is characteristic in both phases, while in mitosis the acrocentric chromosomes 7 and 5 of *aequalis* are distinctive. Chromosome 6 is distinctive in both species, if the secondary constriction can be seen. The first division of meiosis is less useful for separating the species, but the XY bivalent is normally slightly larger in *aquaticus*, and the presence of B chromosomes also indicates *aquaticus*, though it is possible that these chromosomes may be discovered in *aequalis*. The unimpeded pairing of the chromosomes seen in all preparations of first meiotic division indicates that no hybrids have been encountered among the thirty-five specimens examined. All of these come from areas where the ranges of the two species overlap, and twenty-five were from pools where the two occurred together.

Aedeagophores from some of these specimens are shown in Figs. 41–55. The state of affairs they reveal is both surprising and very clear: despite the more or less continuous range of form shown by the aedeagophores of the two species, no hybridization is involved. *H.aequalis* is normally distinct because of its longer parameres with straight outer margins, and relatively shorter basal piece. *H.aquaticus* normally has the parameres shorter, with outer margins curved or even angled, and the basal piece relatively longer. Figs. 41–47 illustrate the range of variation shown by shorter aedeagophores, and Figs. 48–55 that shown by longer ones. In both cases the range of variation shown by *H.aquaticus* exceeds the difference between the two species. *H.aequalis* shows less aedeagal variation. The difficulty in recognizing some of the most similar specimens of the two species is illustrated by Figs. 42 and 43 and Figs. 50 and 51. Recognition can be made more difficult if the genitalia are squashed in being mounted on slides, or if those of recently emerged specimens shrivel on drying.

The range of variation of the *H.aquaticus* aedeagophore exceeds that shown by these French specimens. On the one hand many specimens from the Caucasus have very long basal pieces and shorter parameres (Fig. 31), while others, including the Spanish specimen shown in Fig. 40, have longer parameres.

The only additional variation shown by the *H.aequalis* aedeagophore is illustrated by the very small specimen (the type) shown in Fig. 29.

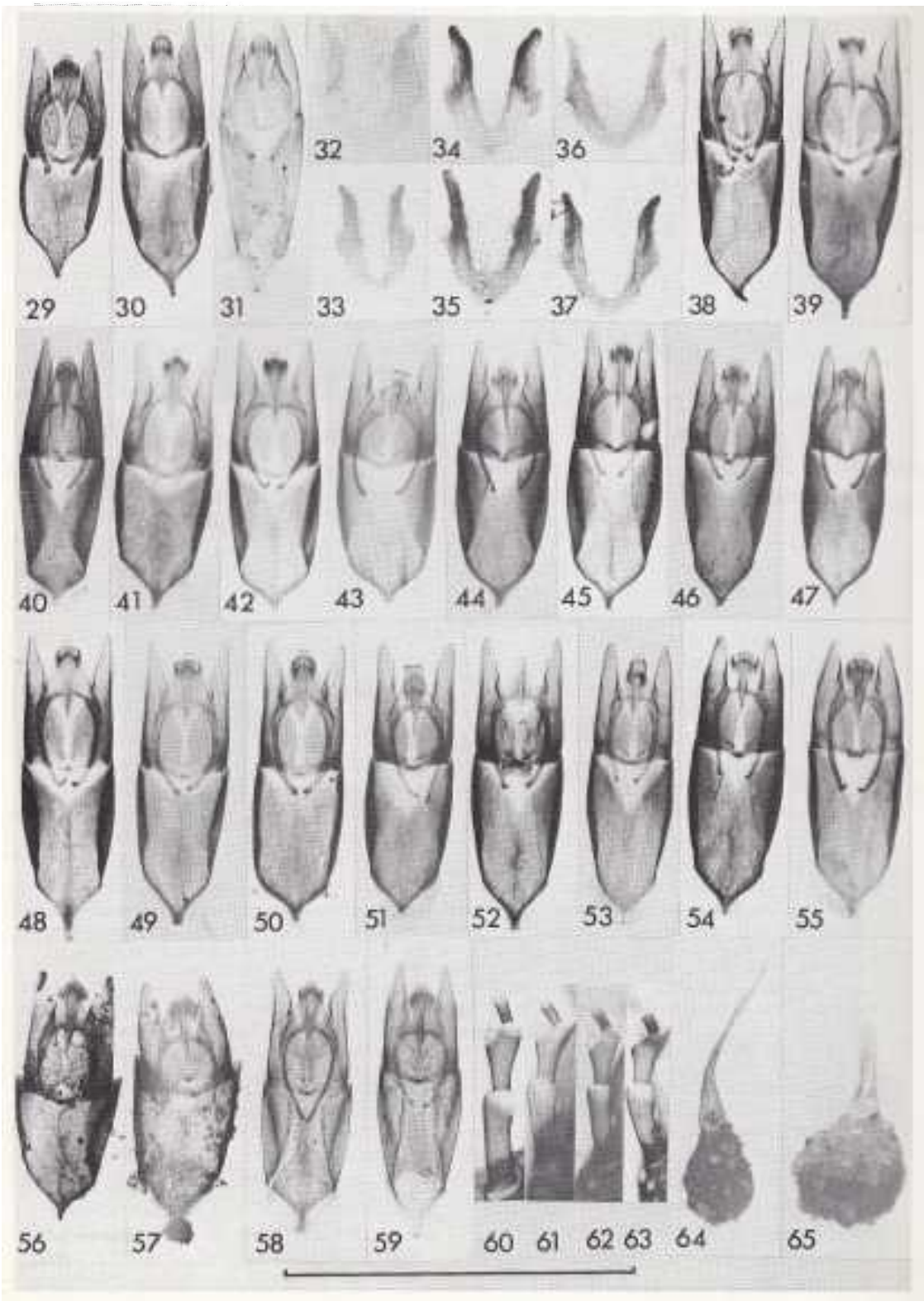
There are no consistently reliable somatic



FIGS. 21–28. *H.aequalis* and *aquaticus*, chromosome preparations from testes of young males, showing features used to recognize the species and to demonstrate the absence of hybridization. Chromosomes of particular importance are labelled. The scale bar represents 5 μm. 21, *H.aequalis* from St Savin, spermatogonial mitosis. 22, *H.aquaticus* from St Savin, spermatogonial mitosis. The Y-chromosome and one other are missing. The large chromosome 6 identifies it as *H.aquaticus*. It cannot be a hybrid as there is no chromosome referable to *H.aequalis* chromosome 5, 7 or X. 23–25, meiosis, first division, diakinesis: 23, *H.aequalis* from Scoury; 24, *H.aquaticus* from Sonzay; 25, *H.aquaticus* from Neuillé-Pont-Pierre. 26–28, meiosis, second division, metaphase: 26, *H.aequalis* from the Brenne, showing the acrocentric X-chromosome; 27, *H.aquaticus* from Sonzay, female haploid nucleus with metacentric X-chromosome not distinguishable from the smaller autosomes; 28, *H.aquaticus* from Neuillé-Pont-Pierre, male haploid nucleus showing one B-chromosome and that the acrocentric chromosome 7 cannot be the X-chromosome.

characters for separating the two species, though *aquaticus* is often smaller and darker than *aequalis*, and usually has the elytra with a fairly extensive pattern of lighter mottling. The elytra of *aequalis* may show this pattern but are often a fairly uniform brown with the dark sutural Λ-mark and dark spots on interstice 6 distinct. These characters appear least reliable in areas where the two species coexist.

The form of the horseshoe-shaped ninth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tergite may be helpful in separating the females. Many *H.aequalis* have the tergite narrow and straight-sided (Fig. 35), while in *aquaticus* it is shorter and with lateral projections, basal to which the sides are concave (Fig. 34). However, both species vary, and Fig. 36 shows *aequalis* in which the tergite approaches the *aquaticus* pattern, while Fig. 37 shows *aquaticus* approaching the *aequalis* pattern.

Types and Synonymy

Study of the various types relevant to the two species, in the light of the range of variation indicated by the chromosomal studies, enables the following synonymy to be established.

H.aequalis Thomson, 1868

aeneus (Degeer, 1774) (Name rejected by the International Commission for Zoological Nomenclature: Rejected name no. 609)

H.aquaticus (L., 1758)

frigidus Graëlls, 1847
? *alpigena* Dalla Torre, 1877
? *dzieduszyckii* Łomnicki, 1894
? *pleistocenicus* var. *obsoletus* Łomnicki, 1894
var. *splendens* Sharp, 1915
natio *caucasicola* Zaitzev, 1946

The following notes explain the synonymy.

H.aequalis. Angus (1970) designated a male lectotype. The aedeagophore is shown in Fig. 29. The form of the parameres, and their size relative to that of the basal piece, establish the identity of the specimen, despite its small size. The lectotype, 3.8 mm long, appears stunted. The female paralectotype is 5.5 mm long. Both specimens are normal *aequalis* in that the elytra are only weakly mottled and the black Λ-marks are distinct.

H.aeneus. A lectotype was designated and figured by Angus (1970). The aedeagophore is typical of *H.aequalis.*

H.aquaticus. The lectotype designated by Angus (1970) is female. The ninth tergite is shown in Fig. 32. The width of the lateral flange and concavity of the basal half of the sides are sufficient to identify the specimen, being outside the observed range of variation of *aequalis.* The paralectotypes are two females, each with well-developed lateral flanges on the ninth tergite, and one male whose aedeagophore is shown in Fig. 30. This aedeagophore is identified as *H.aquaticus* by the curved outer sides of the parameres, which have a blunt angle about a third of the way from the apex. The parameres are as long in relation to the basal piece as is ever the case in this species, but the ratio is the same

FIGS. 29–65. 29–31, aedeagophores: 29, *H.aequalis* Thoms., lectotype; 30, *H.aquaticus* (L.), paralectotype; 31, *H.aquaticus* natio *caucasicola* Zaitzev, lectotype. 32–37, ninth abdominal tergites of ♀♀: 32, *H.aquaticus* (L.), lectotype; 33, *H.frigidus* Graëlls, lectotype; 34, *H.aquaticus* from Valsain, to show the normal appearance in this species; 35, *H.aequalis* from Souvigné, to show the normal appearance in this species; 36, *H.aequalis* from Tain, Sutherland, to show the resemblance to *aquaticus*; 37, *H.aquaticus* from Valsain, to show the resemblance to *aequalis.* 38 and 39, *H.aequalis,* aedeagophores of two males from an egg cocoon laid by the female whose ninth tergite is shown in Fig. 35. 40, *H.aquaticus* from Mount Peñalara, Spain, aedeagophore for comparison with Fig. 30. 41–55, aedeagophores of young males whose identity has been established chromosomally: 41, *H.aequalis* from Scoury; 42, *H.aequalis* from Scoury (chromosomes: Fig. 23); 43, *H.aquaticus* from St Savin (chromosomes: Fig. 22); 44, *H.aquaticus* from Sonzay; 45, *H.aquaticus* from Neuillé-Pont-Pierre; 46, *H.aquaticus* from Sonzay; 47, *H.aquaticus* from Neuillé-Pont-Pierre (chromosomes: Fig. 28); 48, *H.aequalis* from the Brenne; 49, *H.aequalis* from the Brenne (chromosomes: Fig. 26); 50, *H.aequalis* from St Savin; 51. *H.aquaticus* from Sonzay (chromosomes: Figs. 24 and 27); 52, *H.aquaticus* from Neuillé-Pont-Pierre (chromosomes: Fig. 25); 53, *H.aquaticus* from Sorigny; 54, *H.aquaticus* from Neuillé-Pont-Pierre; 55, *H.aquaticus* from Sonzay. 56 and 57, aedeagophores of British Pleistocene fossils: 56, *H.aequalis* from Eemian deposits in Lincolnshire; 57, *H.aquaticus* from mid-Weichselian deposits in Oxfordshire. 58 and 59, aedeagophores of Pleistocene fossils from mid-Weichselian deposits at Starunia in the Western Ukraine: 58, *H.aequalis*; 59, *H.aquaticus.* 60–63, antennae of third instar larvae: 60, *H.aequalis* from St Savin; 61, *H.aequalis* from Pouzol; 62, *H.aquaticus* from Pouzol; 63, *H.aquaticus* from Culan. 64 and 65, egg cocoons: 64, *H.aequalis* from Oxford; 65, *H.aquaticus* from Fontanières. The scale bar represents 1 mm for Figs. 29–63 and 10 mm for Figs. 64 and 65.

as in the specimen from the Peñalara in the Sierra Guadarrama (Spain) shown in Fig. 40. This specimen is identified as *aquaticus* on the basis of many specimens whose form links it to more normally proportioned *aquaticus*, and by the fact that *H.aequalis* does not occur in that part of Spain. The Linnaean specimen may also be compared with the French *aequalis* shown in Fig. 39, which is seen to differ in the absence of any angle on the outer sides of the parameres.

H.frigidus. The female lectotype designated by Angus (1970) is small and soft. Nevertheless, the ninth tergite (Fig. 33) is diagnostic. The type locality is the Laguna de los Pajaros on Mount Peñalara, where, as mentioned above, *H.aequalis* does not occur, though *aquaticus* is abundant.

H.alpigena. Dalla Torre's description of this form as having dark metallic green elytra and living in the Alps is appropriate for *aquaticus* but not *aequalis*. As mentioned by Angus (1970), I have been unable to locate any type material.

H.dzieduszyckii and *H.pleistocenicus* var. *obsoletus.* The type material of these forms was described by Angus (1973a). The types are insufficient to establish whether *H.aquaticus* or *aequalis* (or both) are involved. However, the nearby Pleistocene site at Starunia has more *aquaticus* than *aequalis*, and since both sites must have had a cold continental climate, indicated by *Helophorus jacutus* Popp. (*praenanus* Łomnicki), *H.aquaticus* is the more likely species. Fortunately both these Łomnicki names are younger than both *aquaticus* and *aequalis*, so cannot be used as the valid name for either species.

Var. *splendens.* The male designated lectotype by Angus (1970) is identified as *H.aquaticus* by its relatively short parameres with curved outer sides.

Natio *caucasicola.* Zaitzev described *H.aquaticus* subspecies *aequalis* natio *caucasicola* to cover specimens with rather straight pronotal sides and with the pronotum more conspicuously granulate, which he found widely distributed in high regions of the Caucasus. His collection, in the Zoological Institute, Leningrad, contains eighty specimens from various parts of the Caucasus and Russian Transcaucasus. The specimen here designated lectotype is 4.7 mm long and 2.0 mm wide. The aedeagophore is shown in Fig. 31. The seventh abdominal sternite has the fine teeth characteristic of *H.aquaticus* and *aequalis*. The specimen is labelled 'Val. fl. Ktsia. prov. Tabitschuri. 2.vi.16'. The remaining specimens, including six with the same data as the lectotype, are paralectotypes. All the specimens have been labelled with their type designations.

These Caucasian specimens have the shortest parameres and longest basal pieces of any I have seen. However, the French specimens shown in Figs. 54 and 55 approach their general form, as does the British Pleistocene fossil shown in Fig. 57.

Immature stages

The egg cocoons and larvae of *H.aequalis* are described (as *aquaticus*) by Angus (1973b). Egg cocoons of the two species are shown in Figs. 64 and 65. The egg sac of *H.aequalis* is normally smaller than that of *aquaticus* and usually contains about twelve eggs, as against about fifteen in *aquaticus*. The mast of the *aequalis* cocoon tends to be rather longer than that of *aquaticus*.

The larvae of the two species are very similar, though third instar larvae can often be separated on their sizes, and the shape of the second antennal segment. A series of thirteen French *H.aequalis* larvae range in length from 8.1 to 10.5 mm (excluding the urogomphi), while their head widths, between the eyes, range from 0.86 to 1.0 mm. British specimens, and one from the Lofoten Islands (Norway) appear similar. Nine French *aquaticus* larvae range in length from 7.4 to 10.5 mm, and have head widths from 0.7 to 0.87 mm. Exuviae of Spanish specimens appear similar. The only specimen with a head more than 0.84 mm wide is 10.5 mm long. *H.aequalis* larvae of this length have the head at least 0.95 mm wide. The antennae and legs tend to be more elongate in *aequalis*, and this is most easily seen when comparing the second antennal segment. Most *aequalis* have the segment more or less as in Fig. 60, with the outer limit of the sclerotization of the outer side nearly opposite that of the inner side. In *aquaticus* the apical limit of sclerotiza-

tion of the outer side of the segment is usually opposite a point not far from the middle of the inner side (Fig. 63). However, both species vary, and Figs. 61 and 62 show specimens which cannot be distinguished on the basis of their antennae. Nevertheless, the combination of length, head width and shape of the second antennal segment should allow most specimens to be recognized.

Distribution

H.aequalis is a west european species which seems to require a less continental climate than *aquaticus*. It is widespread in Britain and France, but in Spain is known only from near the Embalse de Ebro, where I collected it in 1974. In Italy I have seen specimens from Rome and Milan, and in the Balkans from Castelnuovo (Dalmatia) and Sliven (Bulgaria). North of the Alps it ranges as far as Wustrow (Mecklenburg), and the Krakow district (Poland). It is widespread in Hungary. In Scandinavia I have seen specimens from the Lofoten Islands and near Trondheim (Norway), and various southern Swedish localities. This is the species figured by Strand (1965). In Finland I have seen males from the Aland Islands, and Uusikaupunki on the mainland. Accurate determination of the limits of its range requires studies by local entomologists.

H.aquaticus has a more eastern and montane distribution. It is absent from Britain, and in France the most north-westerly locality I know is St Léger sur Sarthe (Orne). It is common in the Massif Central, but absent from the Brenne (Indre), though *aequalis* is common there. It is common round Tours, and extends through the Paris area to the Ardennes in southern Belgium. It is common in the Alps and Pyrenees, and in Spain it is abundant in the Cantabrian Mountains, the Sierra Guadarrama and Sierra de Gredos and the Montes de Toledo. The Reitter collection contains one specimen from the Sierra Nevada in the south. In Italy it occurs in the Appennines and in Sardinia. It is widespread in the Balkans, and in Anatolia. North of the Alps it ranges eastwards into Russia, though avoiding the coast as far east as Holstein, from where I have seen specimens from Eutin. It is widespread in Poland and Hungary, and in Russia extends as far east as Ukhta (Komi ASSR), Perm and the Caucasus. It is widespread in Finland, at least as far north as Oulu, but I have not seen any specimens from Norway or Sweden (except possibly the Linnaean types, though these lack locality data). However, I have studied very little material from these countries.

Pleistocene fossils

These species are a common constituent of British Pleistocene faunas studied by G. R. Coope of Birmingham University and his colleagues, the most usual remains being pronota, elytra and seventh abdominal sternites. However, only in those rare instances where aedeagophores are present can the two species be separated. The oldest record for *H.aequalis* is for an aedeagophore from deposits of the Last (Eemian) Interglacial from Tattershall, Lincolnshire, about 120 000 years old. This specimen is shown in Fig. 56. The Tattershall deposits also yielded two further *H.aequalis* aedeagophores among material dating from the warmest phase of the Upton Warren Interstadial Complex, in the middle of the Last (Würm or Weichselian) Glaciation, about 43 000 years ago. A preliminary account of the Tattershall deposits is given by Girling (1974), while detailed studies are reported by Girling (1980).

H.aquaticus is abundant in deposits from Queensford Gravel Pit, near Dorchester on Thames, Oxon., and numerous aedeagophores have been recovered from the material. One of these is shown in Fig. 57. This deposit has been radiocarbon dated at $39{,}300\ ^{+1350}_{-1150}$ BP (Birm-333), and contains an extensive beetle fauna indicative of cold continental conditions. Coope (1976) gives an account of the fauna.

The *Helophorus* fauna of the Pleistocene deposits associated with the woolly rhinoceros found at Starunia in the western Ukraine is described by Angus (1973a). These deposits have been impregnated with a mixture of oil and salt from underlying Miocene rocks, resulting in a unique quality of preservation of the fossils. Both *H.aequalis* and *aquaticus* are present, and the aedeagophores shown in Figs.

58 and 59 illustrate the quality of some of this material. The deposits have been radiocarbon dated at 23 255 ± 775 years BP (SI-642) (Angus, 1973a).

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