Report on a strain of the pathogenic blue-stain fungus *Ceratocystis polonica* with low virulence

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Abstract: The pathogenic blue-stain fungus *Ceratocystis polonica*, associated with the spruce bark beetle *Ips typographus*, was used in an inoculation trial to test the susceptibility of Norway spruce (*Picea abies*). Originally, this study aimed to assess the susceptibility of semi-mature spruces, that showed severe crown thinning due to Sirococcus shoot blight, and to compare their susceptibility to *C. polonica* infection with that of apparently healthy and vigorous spruces. Forty pole-size trees, about 50 years old, were mass inoculated with *C. polonica* at a density of 4 inoculations per dm² in a 80 cm wide band along the circumference of the stem. Unexpectedly, the isolate of *C. polonica*, that was used in this study, displayed low levels of virulence. *Ceratocystis polonica* did not kill any trees five and about 14 months after inoculation. At six trees felled five months after inoculation, the fungus caused on an average only 3.1 % blue-stain and 21.2 % occlusion in the sapwood and killed only 11.0 and 18.6 % of the cambium and phloem, respectively. The low virulence of this isolate of *C. polonica* is inconsistent with most inoculation studies that have been performed previously. The existence of strains of *C. polonica* with low levels of virulence has not been noted in the past and the results of this study suggest that care must be taken when selecting strains for pathogenicity tests.

Zusammenfassung: Der pathogene Bläuepilz *Ceratocystis polonica*, der mit dem Fichtenborkenkäfer *Ips typographus* assoziiert ist, wurde in einem Inokulationsversuch dazu verwendet, die Anfälligkeit von Fichten (*Picea abies*) zu testen. Das ursprüngliche Ziel des Versuches bestand darin, die Anfälligkeit von Fichten im Stangenholzalter, die von einem Trieb-, Zweig- und Aststerben, verursacht durch den Pilz *Sirococcus conigenus*, betroffen waren und starke Kronenverlichtungserscheinungen zeigten, gegenüber einer Infektion durch *C. polonica* abzuschätzen und mit der Anfälligkeit von gesund und vital erscheinenden Fichten zu vergleichen. Vierzig ungefähr 50-jährige Bäume wurden in einer Dichte von 4 Inokulationen pro dm² in einem 80 cm langen Band entlang des ganzen Baumumfanges mit *C. polonica* masseninokuliert. Überraschenderweise übte das in diesem Versuch verwendete Isolat von *C. polonica* nur geringe Virulenz aus und brachte nach fünf und ungefähr 14 Monaten keinen einzigen Baum zum Absterben. Der Pilz verursachte an sechs Bäumen, die fünf Monate nach der Inokulation gefällt wurden, durchschnittlich nur 3,1 % Blauverfärbung und 21,2 % Verstopfung des Splintholzes und tötete lediglich 11,0 % des Kambiums und 18,6 % des Bastes ab. Die geringe Virulenz dieses Isolats von *C. polonica* steht im Gegensatz zu den meisten Inokulationsversuchen, die mit diesem Pilz bisher durchgeführt wurden. Das Auftreten von gering virulenten Stämmen von *C. polonica* war bisher nicht bekannt und die Ergebnisse dieses Versuches regen an, Isolate für Pathogenitätstests sorgfältig auszuwählen.

The blue-stain fungus *Ceratocystis polonica* (SIEMASZKO) C. MOREAU is associated with the aggressive spruce bark beetle *Ips typographus* L. in Europe (SIEMASZKO 1939; SOLHEIM 1986, 1992; HARDING 1989a; VIIRI & WEISSENBERG 1995; KROKENE & SOLHEIM 1996; KIRISITS 1996; GRUBELNIK 1998) and with its Asian relative, *Ips typographus* f. *japonicus* NIIJIMA in Japan (YAMAOKA & al. 1997). Recently, two other bark beetle species colonizing Norway spruce, *Ips duplicatus* SAHLB. (KROKENE & SOLHEIM 1996) and *Ips amitinus* EICHH. (KIRISITS & al. 1998) were recognized as important insect associates of *C. polonica* in Europe. Other European spruce bark beetles such as *Pityogenes chalcographus* L., *Polygraphus poligraphus* L., *Hylurgops palliatus* GYLL. and *Hylurgops glabratus* ZETT. may casually act as vectors for *C. polonica*, but do not appear to be consistently associated with this phytopathogenic blue-stain fungus (KROKENE & SOLHEIM 1996; KIRISITS 1996; KIRISITS, unpubl. data). *Ceratocystis polonica* displays high levels of virulence to Norway spruce, *Picea abies* (L.) KARST., and is able to kill trees that have been mass inoculated with it (HORNTVEDT & al. 1983; CHRISTIANSEN 1985a, b; SOLHEIM 1988; KROKENE & SOLHEIM 1998; KIRISITS 1998). Due to its high levels of virulence to Norway spruce, the fungus is suspected to aid its bark beetle vectors in overcoming the defense systems of the host trees (CHRISTIANSEN & al. 1987, KROKENE 1996).

Ceratocystis polonica has been used in several mass inoculation experiments to assess the resistance of Norway spruce and to evaluate various aspects of the pathogen - host tree interactions (e.g., CHRISTIANSEN 1985b, CHRISTIANSEN & ERICSSON 1986, CHRISTIANSEN 1992, CHRISTIANSEN & FJONE 1993, FRANCESCHI & al. 1998, BRIGNOLAS & al. 1998). In spring 1998 an experiment with *C. polonica* was initiated in order to assess the susceptibility of Norway spruce, that were heavily affected by shoot, twig and branch die-back, caused by the fungus *Sirococcus conigenus* (DC.) CANNON & MINTER, and to compare their resistance with that of healthy Norway spruces. Studies by CHRISTIANSEN & FJONE (1993) revealed, that artificial pruning enhances the susceptibility of Norway spruce to infection by *C. polonica*. In the present study we wished to investigate, whether partial defoliation by Sirococcus shoot blight has similar effects on susceptibility. A further aim was to examine the effect of fertilizer application on the resistance of Norway spruce to *C. polonica* infection. Unexpectedly, the isolate of *C. polonica*, that was used in this study, displayed low levels of virulence and we report on this observation here.

Table 1. Characteristics of Norway spruce trees inoculated with *Ceratocystis polonica* at the study site "Kobernaußerwald". Values are means of 20 trees (± standard deviation). [1]Disease category refers to shoot, twig and branch die-back primarily caused by *Sirococcus conigenus*. [2]diameter at breast height. [3]Crown thinning classes were assessed according to POLLANSCHÜTZ & al. (1985). The crown thinning classes correspond to the following values of crown defoliation: *1* 0-15 % defoliation, *2* 15-30 %, *3* 30-50 %, *4* above 50 %.

Disease category[1]	DBH[2] [cm]	Crown thinning class[3]
Heavily affected	19.1 ± 1,8	1.1 ± 0,2
Not affected	18.4 ± 1,6	3.5 ± 0,5

Material and methods

Study area: The inoculation experiment was performed in an approximately 50 year-old, pole stand of Norway spruce located at the region "Kobernaußerwald" in Upper Austria. The area is owned by the Austrian Federal Forests and located within the Forest administration Mattighofen (Forest District

Frauschereck, sub-compartment 168a). The spruce stand covers an area of about 5 ha and lies 600 m s. m. on a west exposed, 15° inclined slope. According to the classification of the forest ecoregions of Austria (KILIAN & al. 1994), the site belongs to the forest ecoregion 7.1 ("Nördliches Alpenvorland - Westteil"). The stand consists of almost pure Norway spruce with negligible admixture of Silver fir (*Abies alba* MILL.) and European Beech (*Fagus sylvatica* L.).

The study site is one of several that form part of the Special Research Program "Forest Ecosystem Restoration", which considers various aspects of the restoration of secondary spruce forests. At the spruce stand used in this study, the cause and impact of shoot, twig and branch die-back occurring at semi-mature and mature Norway spruce trees has been investigated in the past (ANGLBERGER & HALMSCHLAGER, unpubl. data). This disease occurs in different parts of Upper Austria and is suspected to be primarily caused by the mitosporic fungus *Sirococcus conigenus*, that leads to blight of the current year shoots (NEUMÜLLER 1994, ANGLBERGER 1998, HALMSCHLAGER & al. 1998). Subsequent fungal infections may give rise to twig and branch die-back progressing from the tips of the branches towards the inner crown and consequently lead to significant crown thinning and increment losses (NEUMÜLLER 1994, ANGLBERGER 1998, HALMSCHLAGER & al. 1998). In 1994, one part of the spruce stand was fertilized with 1700 kg/ha of the Mg-based fertilizer Biomag® M3B (Veitsch-Radex AG). This treatment was done as a control measure, as nutritional imbalance, especially lack of Mg, is thought to be an important factor leading to disease (ANGLBERGER 1998, HALMSCHLAGER & al. 1998).

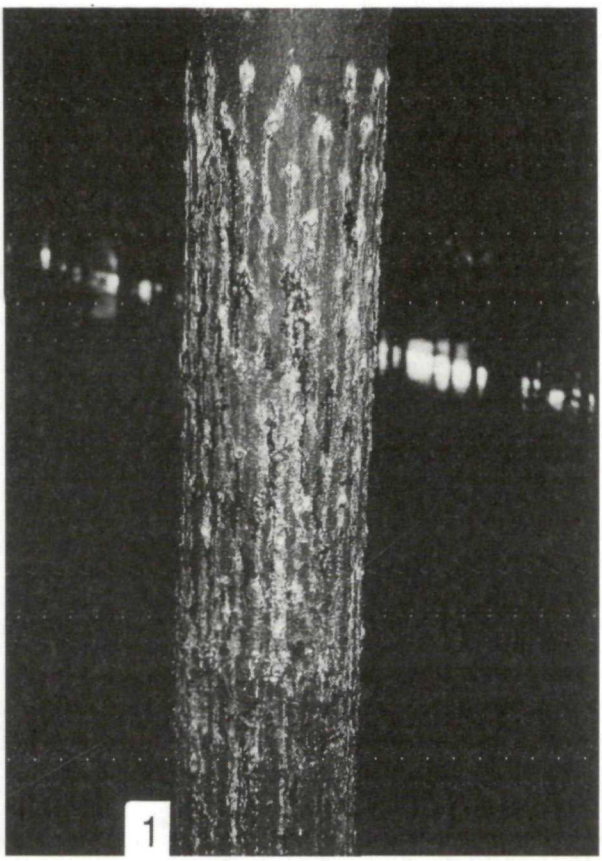

Fig. 1. Intensive resin exudation from the inoculation wounds of tree no. 29 about 14 months after mass inoculation with *C. polonica*.

Trees for inoculation: In April 1998, 40 dominant Norway spruce trees were selected for inoculation (Table 1). Half of the trees were affected by shoot, twig and branch die-back, and thus showed severe crown thinning. The other twenty trees were not affected by the disease and appeared to be healthy and vigorous based on general appearance and crown condition. At the time of selection, the trees were visually inspected for the incidence of shoot, twig and branch die-back using binoculars. The severity of the disease was determined on each tree by estimating the crown thinning class according to POLLANSCHÜTZ & al. (1985). Diseased trees belonged to crown thinning classes 3 and 4, corresponding to a crown defoliation of 30-50 % (class 3) and above 50 % (class 4), compared with apparently healthy Norway spruces. Healthy trees belonged to crown thinning class 1 (crown defoliation of 0-15%), with the exception of one tree, that had crown thinning in class 2, corresponding to a crown defoliation of 15-30 %. Half of the trees of each disease category (affected - not affected) were located at the fertilized part of the stand, the others were growing in the unfertilized area. At the time of selection, diameters (DBH) of all trees were measured and dry branches up to 2 m above ground level were removed.

Fungal isolate and inoculation procedures: The strain of *C. polonica* (isolate no. Ko/28/5 from the culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection, Vienna) used in this experiment was isolated in July 1997 during the course of a survey of the blue-stain fungal mycobiota of *Ips typographus* in Austria (GRUBELNIK 1998). In this study fungal isolations were done by inoculating beetles into Norway spruce logs and re-isolating fungi about six weeks later from discolored phloem and sapwood (GRUBELNIK 1998). The isolate "Ko/28/5" of *C. polonica* was isolated from the sapwood of a log inoculated with an individual of *I. typographus*, that had been collected at the area "Kobernaußerwald", only a few kilometers away from the site of the present inoculation study.

From April 30th to May 3rd, 1998 the trees were mass inoculated with *C. polonica* at a density of four inoculations per dm² in a 80 cm wide band along the circumference of the stem at a height between 1 and 1,8 m above ground level. Thus, a tree with a DBH of 20 cm received about 200 inoculations. Based on previous experiments performed in Norway (CHRISTIANSEN 1985b, BRIGNOLAS & al. 1998), this dosage was thought to be appropriate to overwhelm the defense systems of susceptible trees, but not those of disease tolerant trees. Prior to treatment, the inoculation sites were marked within the inoculation belt, using a template to ensure even placement of inoculum. Trees were inoculated by removing a bark plug with a 7 mm cork borer, inserting inoculum into the wounds, and replacing the bark plug. Tools were rinsed in 96 % ethanol and flame sterilized between inoculation of different trees. Inoculum consisted of plugs of malt agar (2 % malt, 1.6 % agar) bearing mycelium from 9 to 12 days old cultures of *C. polonica*. The inoculum was cut a few days prior to inoculation with a sterile 5 mm diameter cork borer under sterile conditions. No control treatment using sterile malt agar as inoculum was included in this study, because a variety of experiments has shown, that such control inoculations cause only small amounts of necrosis in the phloem and little desiccation in the sapwood (HORNTVEDT & al. 1983, SOLHEIM 1988, KROKENE & SOLHEIM 1998).

Inspection of the trees and assessment of virulence: Trees were first inspected after 148 days on September 28th, 1998. On September 28th and 29th, six of the 40 treated trees were felled. After felling, tree height and tree age at stump height were recorded and a 1 m long stem section, including the area of inoculation was cut and taken to the laboratory. The outer bark around 50 inoculation sites was removed from each stem section. Length and width of the lesions in the phloem around each inoculation point was measured. At tree no. 4 the lesions frequently (41.7 % of those measured) had coalesced within the inoculation area. Thus, only 24 lesions located at the uppermost and the lowermost inoculation rings were measured from the centre of the inoculation wound in one direction, away from the inoculation belt. The length measurements of the other five trees were halved in order to compare them with those of tree no. 4.

Two 5 to 10 mm thick discs were cut from the stem sections 20 cm below the upper margin and 20 cm above the lower margin of each inoculation belt, respectively. Based on translucent appearance, the sapwood/heartwood border as well as desiccated (occluded), blue-stained and apparently healthy (translucent) sapwood areas were marked on each stem disc. Additionally, dead and live cambium areas were marked along the circumference of the stem discs. The various areas of each stem disc were traced onto sheets of transparent paper and were digitized with an optical scanner. The area of

Figs. 2 and 3. Stem discs of trees no. 4 and no. 14, felled 148 days after mass inoculation with *C. polonica*. Most of the sapwood is still functional and only small parts are blue-stained or desiccated due to the influence of the fungus inoculated. Tree no. 4 was most affected of the six trees examined. *h* heartwood, *b* blue-stained sapwood, *d* desiccated sapwood, *s* water-holding (fresh) sapwood.

heartwood, total sapwood, healthy, desiccated and blue-stained sapwood as well as length of dead and live cambium along the circumference of the discs was later determined with image analysis software (LUCIA 32G Version 4.10, Laboratory Imaging, Czech Republic, http://www.lim.cz) and values of percentage of blue-stained and desiccated sapwood and percent dead cambium were computed. From the central 40-cm stem section the outer bark was removed and necrotic lesions in the phloem were traced in a 30 x 20 cm area onto sheets of thin plastic. The plastic sheets were photocopied and total area of necrotic phloem was determined following the same procedure as described above for areas of blue-stained, desiccated and healthy sapwood. On June 22nd, 1999, about 14 months after inoculation, the remaining trees were again inspected for the presence of symptoms of decline.

Results

First inspection: When first inspected five months after inoculation, all trees had reacted with intensive resin exudation from the inoculation sites (Fig. 1). None of the trees showed external symptoms of fungal infection such as foliage browning or needle drop. Inoculation with *C. polonica* gave rise to little damage both in the phloem, cambium and sapwood of the six trees examined (Table 2). Necrotic lesions around the inoculation sites were small and did not coalesce, with the exception of those in tree no. 4 (Fig. 4). This is also reflected in the measurements of killed cambium and killed phloem (Table 2). Only three trees had small proportions of blue-stained sapwood and the amount of occluded (blue-stained and desiccated) sapwood area was generally low (Table 2, Figs. 2 and 3).

The isolate of *C. polonica* used in this study caused lesions that were an average of 15.9 mm long (half lesions length) and 12.6 mm wide, gave rise to an average of 3.1 % blue-stain and 21.2 % occlusion in the sapwood and killed an average of 11.0 and 18.6 % of the cambium and phloem, respectively. Traumatic resin ducts incorporated in a false growth ring within the current annual ring of sapwood (CHRISTIANSEN & FJONE 1993, CHRISTIANSEN & al. 1999) were seen on the stem discs of all six trees.

Second inspection: In June 1999 all remaining trees were alive and none showed symptoms of decline. The only exception was a severely diseased tree of low vigour, with only a few living branches. However, this tree showed intensive resin exudation from the inoculation sites typical for trees that are tolerant to the fungal inoculation. We thus assume, that the inoculation of *C. polonica* was not the primary cause of decline in this tree.

Discussion

None of the treated trees showed signs of decline at the first inspection, five months after inoculation. Thus, the original aims of the study were abandoned and we have subsequently focussed our attention of the activities of an unusual isolate of *C. polonica* that has low levels of virulence. Measurements pertaining to fungal infection (Table 2) on six of the 40 inoculated trees, as well as the observation that no tree was killed about 14 months after inoculation, confirmed the fact that the isolate chosen for study had unusually low virulence.

The low level of virulence of the isolate of *C. polonica* used in this study is inconsistent with most mass inoculation studies that have been performed with this fungus in the past (e.g., HORNTVEDT & al. 1983; CHRISTIANSEN 1985a, b, 1992; CHRISTIAN-

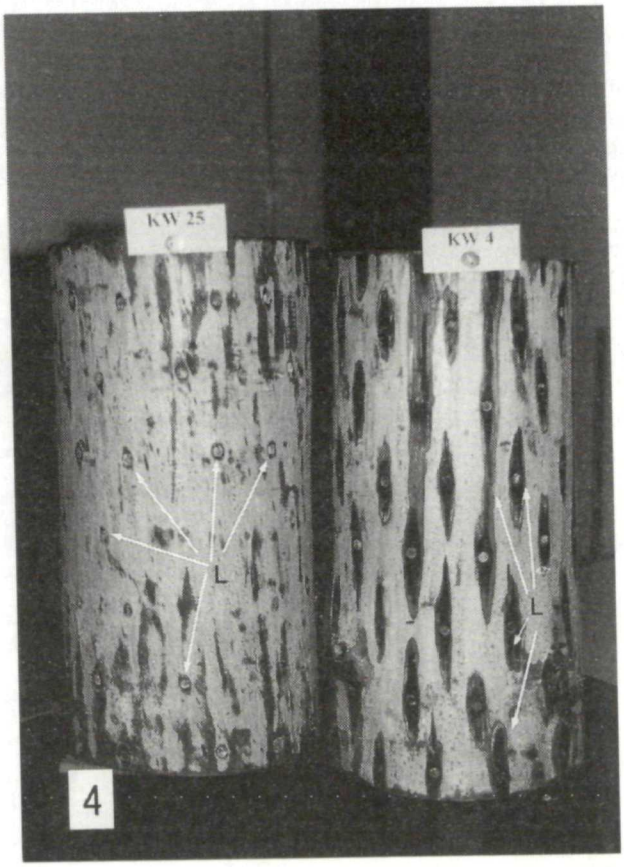

Fig. 4. Stem sections of tree no. 4 ("KW 4") and no. 25 ("KW 25"), felled 148 days after mass inoculation with *C. polonica*. The outer bark was removed from the stem sections and necrotic lesions are visible in the phloem. Dimensions of the lesions on the two trees show considerable variation. Those of tree no. 25 were amongst the smallest and those of tree no. 4 were the biggest of the six trees that were felled in September 1998 (see also Table 2). A few necrotic lesions (*L*) are marked with arrows.

SEN & ERICSSON 1986; CHRISTIANSEN & SOLHEIM 1990; CHRISTIANSEN & FJONE 1993; HORNTVEDT 1988; HORNTVEDT & SOLHEIM 1991; KROKENE & SOLHEIM 1998; KIRISITS 1998). Pathogenicity of *C. polonica* to Norway spruce was first demonstrated by HORNTVEDT & al. (1983). These authors were amongst the first, who applied the mass inoculation technique. Here, trees receive multiple fungal inoculations in order to overcome their defense systems. Consequently, CHRISTIANSEN (1985a, 1985b) revealed, that the host response of Norway spruce to *C. polonica* is dose-dependent. At a certain dose of fungal inoculation, the defense systems are overwhelmed and trees may die. CHRISTIANSEN (1985b) showed, that eight inoculations per dm² in a 60 cm long belt is a highly lethal dose for Norway spruce, whereas at a dose of four inoculations per dm² in a 60 cm long belt only about half of the trees showed intensive blue-stain. Since the inoculation dose used in the present study lies between the doses mentioned above, we would have expected considerably greater lesion development and much

more sapwood occlusion on the trees, especially on those, that were diseased and not vigorously growing. Apparently, the defense systems of these trees were not overwhelmed by the fungus and at the stem discs traumatic resin ducts (CHRISTIANSEN & al. 1999), a characteristic of trees resisting inoculation of *C. polonica*, were present.

Despite the findings of this study, we cannot with certainty exclude the possibility, that the host resistance of the trees contributed to the outcome of this experiment since no other, virulent isolate of *C. polonica* was used in this study for comparative purposes. In order to exclude this possibility, the isolate was again used in an inoculation study in 1999 to compare its virulence with another isolate, that has already been shown to be virulent to Norway spruce. The latter experiment is, as yet, not complete, and its outcome will be presented elsewhere.

The isolate used in this study had been stored at about +4 °C for one year on malt extract agar in a glass tube before it was used. It is unlikely, that these storage conditions had an influence on the virulence of the isolate. HORNTVEDT & SOLHEIM (1991) did not find differences in the virulence of freshly isolated strains compared to ones, that had been stored for one and three years, respectively. Likewise, an isolate of *C. polonica*, that had also been stored in this way, caused intensive blue-stain in the sapwood of *Picea abies* in another inoculation trial performed in 1998 in Austria (KIRISITS, unpubl. data).

Due to differences in methods and timing of inoculation it may be difficult to compare the results of this study with those performed with *C. polonica* previously. However, the results of a few inoculation experiments might have been influenced by the comparatively low virulence of the isolates of *C. polonica*. For example, *C. polonica* did not cause any blue-stain and only little desiccation in the sapwood in the study performed by SOLHEIM (1988) and this might have been linked to low levels of virulence in the isolate chosen for inoculation. Likewise, one isolate of the fungus gave rise to only desiccation, but no blue-stain in the sapwood in the experiments of KROKENE & SOLHEIM (1998). Finally, the low proportion of blue-stain and sapwood occlusion caused by *C. polonica* in a Danish study (HARDING 1989b) is in contrast to most of other experiments mentioned above. The results of this study, and perhaps those of various previous authors suggest that isolates of *C. polonica* with low virulence occur in collections. Special care must thus be taken to select appropriate isolates prior to conducting trials to determine the ecological relevance of this fungus.

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Table 2. Tree characteristics of the six trees that were harvested in September 1998 and measurements of virulence at these trees following mass inoculation with *C. polonica*. [1]Diameter at breast height. [2]Tree age was recorded at stump height. [3]Disease category refers to incidence of shoot, twig and branch die-back caused by *Sirococcus conigenus*: + diseased tree, - healthy tree. [4]Crown thinning class was assessed according to POLLANSCHÜTZ & al. (1985). Crown thinning classes 1 and 4 correspond to a crown defoliation of 0-15 % and above 50 %, respectively. [5]Measurements of the half length of phloem necroses are given. Sample size for phloem necrosis is 50 per tree, except for tree no. 4, where only 24 lesions were measured.

Tree-no.	DBH [1] [cm]	Tree age[2]	Tree height [m]	Disease category[3]	Crown thinning class[4]	Phloem necrosis[5] [mm]		% Sapwood area		% Killed cambium	% Killed phloem
						Length	Width	Blue-stained	Blue-stained and desiccated		
4	19.7	46	17.3	+	4	34.0	15.9	7.2	34.3	32.4	43.6
14	16.9	47	14.2	+	4	15.3	12.2	2.1	17.3	12.2	13.3
15	16.0	45	13.8	+	4	6.7	10.9	0.0	13.9	1.4	9.2
17	18.8	48	19.0	-	1	10.7	11.5	0.0	16.5	4.1	13.3
25	18.9	47	18.4	+	4	6.8	12.0	0.0	21.6	0.0	5.5
26	17.9	44	15.7	+	4	21.8	13.3	9.0	23.7	16.0	26.4